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Adipocytokines, Energy Balance, and Cancer

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Preface

Adipose tissue, composed of adipocytes, stromal cells, immune and inflammatory cells, and vascular components, was initially considered a depot, storing fuel in the form of fats during times of energy excess and providing fuel for body functions in time of energy needs. Regulation of storage, release, and utilization has been found to be due to the interaction of a complex array of signaling factors that may act locally and/or systemically by autocrine, paracrine, and endocrine mechanisms. These include a variety of growth factors, hormones, and other regulatory agents synthesized in brain, endocrine organs, muscle, gastrointestinal, and adipose tissue itself. When synthesized and secreted in adipose tissues, these factors are designated adipocytokines; they have major physiologic and behavioral effects and their concentrations fluctuate in response to expansion and concentration of adipose tissue, body composition, and a variety of other physiologic signals. In addition to the role of adipocytokines in regulating multiple aspects of energy metabolism, excess adipose tissue has been identified as a source of chronic low-grade inflammation leading to the synthesis of a variety of proinflammatory signals. It is now apparent that in addition to their role in regulating energy metabolism, adipocytokines also contribute significantly to many of the comorbidities associated with the current worldwide obesity pandemic including diabetes, cardiovascular disease, and cancer.

The goal of this volume on *Adipocytokines*, *Energy Balance*, *and Cancer* is to describe recent advances in understanding adipocytokines, their physiologic role in normal regulatory processes, and their mechanistic contributions to the multiple malignancies that increase in response to changes in body mass. These mediators and their effects will be discussed also as potential interventional targets for cancer prevention and control.

This volume begins with an analysis of the components and molecular biology of adipose tissue and differences in activities and interactions related to body distribution. Subsequent chapters deal with both the physiologic and pathologic functions of the major adipocytokines including their regulatory role, their role in energy metabolism, and their role in cancer etiology, promotion, and progression. Adipocytokines to be discussed include Leptin, Adiponectin, Visfatin, Retinol-Binding Protein, Apelin, Macrophage Chemotactic Factor-1, Plasminogen Activator Inhibitor-1,

Chemerin, C-Reactive Protein, and Resistin as well as Gastrointestinal Regulatory Peptides including Ghrelin, Glucagon-Like Peptide, and others.

This volume on *Adipocytokines, Energy Balance, and Cancer* is unique in examining in depth the multiplicity of adipocytokines, their physiologic regulatory mechanisms, and their pathologic role in promoting cancer. We have been fortunate to assemble chapters authored by an international group of authors with expertise in the multiple signaling molecules, and we are grateful for their contribution to this volume.

Chapter 1, written by Caner Saygin and Ofer Reizes (Case Western Reserve University Lerner College of Medicine) and Nathan A. Berger (Case Western Reserve University School of Medicine), provides an overall introduction to adipose tissue as a secretory organ and identifies the major adipocytokines and their functions. Chapter 2 by V.B. O'Leary (Helmholtz Zentrum Munich Institute of Radiation Biology) and J.P. Kirwan (Case Western Reserve University Lerner College of Medicine) focuses on molecular aspects of adiponectin secretion, its regulatory roles in cellular signaling activities, and its contribution to tumor metabolism. In Chap. 3, Margot Cleary (University of Minnesota) and Marta Torroella-Kouri (University of Miami School of Medicine) focus on leptin and its association with cancer while in Chap. 4, Neeraj K. Saxena (University of Maryland School of Medicine) and Dipali Sharma (Johns Hopkins University School of Medicine) discuss cancer therapeutic strategies targeted at leptin. Chapter 5, written by Daniel C. Berry (University of Texas Southwestern Medical Center) and Noa Noy (Case Western Reserve University Lerner College of Medicine), provides a comprehensive analysis of the role of Retinol Binding Protein 4 in Vitamin A metabolism and its relation to diabetes and cancer. In Chap. 6, Maria Dalamaga and Gerasimos Socrates Christodoulatos (University of Athens) describe the interesting intra- and extracellular relation of Visfatin/Nicotinamide Phosphoribosyl Transferase and its relation to obesity and cancer. Chapter 7, written by Stefanie Kälin (Technical University Munich) and Roland Kälin (Ludwig-Maximilians University Munich), provides an introduction to Apelin, one of the most recently identified adipocytokines and its role in physiologic and pathologic conditions especially tumorigenesis. E. Angela Murphy (University of South Carolina) in Chap. 8 provides insight into several novel adipocytokines including Monocyte Chemotactic Protein-1, Plasminogen Activator Inhibitor-1, and Chemerin and their potential dysregulated effects on obesity and cancer. The contribution of resistin to insulin resistance, obesity, and cancer is described in Chap. 9, written by Zhenzhen Zhang, Jackilen Shannon (Oregon Health and Science University), and Hanrui Zhang (Perelman School of Medicine, University of Pennsylvania). C-Reactive Protein, generally considered an acute phase reactant but also categorized as an adipocytokine associated with inflammation and cancer, is discussed by Helen Swede (University of Connecticut School of Medicine) and Dejana Braithwaite (University of California San Francisco). In addition to the host of adipocytokines described in preceding chapters, there is yet another series of peptide signaling molecules, the gastrointestinal regulatory peptides, secreted from the GI tract that regulate hunger, satiety, gastrointestinal motility, and other physiologic functions. Their activities and impact on obesity and cancer are described in Chap. 11 by Debora Bruno and Michael Wolfe (Case Western Reserve University School of Medicine).

Overall, this volume on *Adipocytokines, Energy Balance, and Cancer* provides a valuable addition to the Energy Balance and Cancer series to inform readers of the most recent information on both the normal and disease promoting activities of these adipocyte-derived signaling molecules. This volume should be useful to all students, researchers, and clinicians involved or interested in mechanisms by which obesity contributes to its associated comorbidities, especially cancer, and should serve as a foundation to stimulate research to develop adipocytokine-targeted interventions to disrupt these processes.

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Chapter 1
Adipocytes, Adipocytokines, and Cancer

Caner Saygin, Ofer Reizes, and Nathan A. Berger

Abstract Obesity is now a well-established promoter of cancer progression and decreased overall patient survival. Ever since the association between obesity and cancer was appreciated, adipose tissue, adipocytes, and secreted fat-derived factors have been a focus of the mechanism underlying this link. Adipose-secreted factors cytokines are referred to as adipocytokines and represent the group of molecules thought to link adipose or fat cells to initiation and promotion of various cancers. There are over 20 identified adipokines, of which, a subset has been implicated in cancer. In this chapter, we will provide a concise review of the current literature on the subset of adipose-derived factors linked to cancer.

Keywords Adipocytes • Leptin • Adiponectin • Visfatin • Resistin • Apelin • Chemerin • Omentin • Nesfatin • Vaspin • Retinol-binding protein-4

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Adipose Tissue

Adipose tissue is a loose connective tissue that consists primarily of adipocytes. These cells are terminally differentiated from a progenitor population called preadipocytes. Adipose tissue also contains fibroblasts, blood vessels, and immune cells that collectively constitute the stromal fraction. Adipose tissue is distributed throughout the body, and its development, growth, and energy storage capacity are controlled by genetic, environmental, epigenetic, and pharmacological factors [1]. Adipose tissue was long thought to have a very limited physiological role, relegated to energy storage, support, and heat insulation. However, accumulating evidence indicates that adipose tissue is in fact a multifaceted organ with endocrine, metabolic, and immune regulatory functions. Moreover, adipocytes, which constitute over 80 % of the cells and 90 % of the volume, have high metabolic activity and influence normal homeostasis via secretion of adipocytokines that activate various autocrine, paracrine, and endocrine pathways. This chapter will survey the adipocytokines and their physiological functions and role in cancer.

While adipose tissue is widely distributed, it is not homogenous across the body and exhibits distinct functional and metabolic profiles based upon localization such as abdominal, subcutaneous, and visceral adipose sites. Adipose tissue can be further classified based on adipocyte coloration and is divided into brown, white, and beige [1]. Brown adipose tissue is multilocular (i.e., each adipocyte contains multiple fat globules), expresses uncoupling proteins (e.g., thermogenin), which enable non-shivering thermogenesis, and declines in abundance as the person ages [2]. On the other hand, white adipose tissue is unilocular and is the primary energy store and lacks expression of uncouplers. In addition, there is a unique yellow adipose tissue found in bone marrow, which fills trabecular cavities and is involved in systemic energy regulation and management of insulin sensitivity [3].

Obesity is defined as expansion of fat tissue as a consequence of both hypertrophy and hyperplasia of adipocytes. Current prevalence of obesity in the USA is estimated to be 34.9 % among US adults [4]. In the mid-1990s, no US state had a prevalence of greater than 15 %, but over the past 20 years the frequency has risen dramatically with all US states reporting greater than 20 % prevalence. In addition to its well-known general association with various chronic diseases including diabetes and cardiovascular disease, recent studies highlight the association of obesity with development, recurrence, and chemoresistance of multiple cancers [5]. Approximately 20 % of all cancers are caused by excess weight, and recurrence rates are higher among obese cancer survivors compared to lean patients [5–7]. In addition, the Million Women Study has shown that 50 % of cancers in postmenopausal women are associated with obesity [8]. Among the proposed mechanisms linking obesity to high cancer risk are the adipose-derived cytokines and their signaling pathways including consequent insulin resistance/hyperinsulinemia and inflammation/oxidative stress. In this chapter, we review the impact of adipose tissue on tumorigenesis and cancer progression by highlighting the major adipocytokine pathways. Detailed descriptions of these adipocytokines and their specific roles in tumor biology will be discussed in subsequent chapters.

Adipocytokines and Cancer

"Adipokines" or "adipocytokines" represent over 20 different hormones and signaling molecules secreted from adipocytes, which act both locally in their microenvironment as either autocrine or paracrine factors, as well as at distant sites in an endocrine manner as hormones (Table 1.1). Adipokines are implicated in regulation of multiple physiologic processes including but not limited to energy balance (e.g., glucose homeostasis, insulin resistance), angiogenesis, blood pressure, and inflammatory processes [9].

Table 1.1 Summary of the mechanism of action and effects of adipocytokines

	Mechanism of action	Physiological effects	Pathophysiological effects
Leptin	JAK/STAT, MAPK, PI3K pathways	Inhibits hunger/ stimulates satiety	Increased cell proliferation, growth, survival, angiogenesis, invasion/migration, inflammation, and dysregulated cytokine signaling
Adiponectin	AMPK and PPAR-alpha pathways, increased ceramidase activity	Glucose and lipid homeostasis, insulin sensitivity	Hypoadiponectinemia causes insulin resistance and loss of inhibitory effect on cell proliferation, survival, migration, and inflammation
Visfatin	ERK, MAPK, and cytokine pathways	B cell and vascular smooth muscle maturation, NAD biosynthesis, insulin mimetic (?)	Increased cell survival, cytokine production, migration, increased antioxidative enzymes
Resistin	PI3K, MAPK, and NF-kB pathways	Energy homeostasis	Increased inflammation, cell survival, adhesion, migration and metastasis, insulin resistance (?)
Apelin	G-protein-coupled receptor, PI3K, and ERK pathways	Blood pressure control and angiogenesis, histamine and insulin release, fluid homeostasis	Increased cell proliferation, migration, survival, lymphangiogenesis, and angiogenesis
Chemerin	G-protein-coupled receptor, MAPK/ ERK pathways	Adipocyte differentiation, chemoattractant	Increased inflammation and invasion, recruitment of immune cells
Omentin	Akt, AMPK/ eNOS pathways	Modulation of insulin action, increased cell differentiation and suppression of inflammation	Promotes apoptosis, glucose intolerance (?)
Nesfatin	AMPK, Akt	Anorexigenic, glucose metabolism, insulin sensitivity	Promotes apoptosis

(continued)

Table 1.1 (continued)

	Mechanism of action	Physiological effects	Pathophysiological effects
Vaspin	AMPK, Akt	Glucose and lipid metabolism, anorexigenic, improves glycemia	Impaired insulin sensitivity, ER stress
Retinol-binding protein (RBP)-4	Carries retinol to peripheral tissues, JAK/STAT	Retinol transport, fuel sensing	Insulin resistance (?)
Cytokines			
TNF-alpha	JNK-fos, MAPK, NF-kB	Immune responses, cell death	Pro-inflammatory, insulin resistance, transcription of cell survival genes vs. stimulation of apoptosis
IL-6	JAK/STAT, SOCS1 and 3	Immune responses	Pro-inflammatory, impaired insulin signaling

In 1993, the first adipocyte-derived protein described was TNF-α, which is a pro-inflammatory cytokine and elevated in obese rodents and in humans [10]. Subsequent studies greatly emphasized the link between adipose tissue and inflammation, elucidating the role of other adipose-secreted cytokines (i.e., interleukin-6; IL-6 and IL-1B), chemokines (i.e., monocyte chemoattractant protein [MCP]-1), and special adipokines, including leptin, which suppresses appetite and is pro-inflammatory as well as adiponectin, which stimulates appetite and is anti-inflammatory [11].

In 1994, a team led by Jeffrey Friedman at the Rockefeller University identified the gene responsible for the *obese* (*ob*/*ob*) mutation in mice and named the hormone leptin [12]. The mutant mice exhibit early-onset obesity as a consequence of excess feeding and reduced metabolism. Early physiological analyses suggested a circulating factor was deficient in *ob*/*ob* mice [13]. The discovery of leptin as the circulating factor secreted from adipose tissue solidified adipose tissue as an endocrine tissue. The studies provided a molecular link for communication between adipose tissue and the central nervous system.

It has long been known that certain common cancers including but not limited to breast, colon, endometrium, kidney, prostate, and pancreas have a strong association with obesity [5]. Epidemiological studies investigating the relationship between specific circulating adipocytokines and cancer began in the late 1990s. It is now well appreciated that adipokines constitute an important link between obesity and high cancer risk or cancer mortality via their specific effects on cell proliferation, metabolism, survival, migration and invasion, angiogenesis, and tumor microenvironment (e.g., inflammation) (Fig. 1.1).

Obesity is a global epidemic and a serious health concern that is accepted as a major risk factor for the development of various cancers [7]. Moreover, obesity increases the likelihood of dying from cancer and represents a poor prognostic factor for recurrence as well as associated with chemotherapy resistance. The most

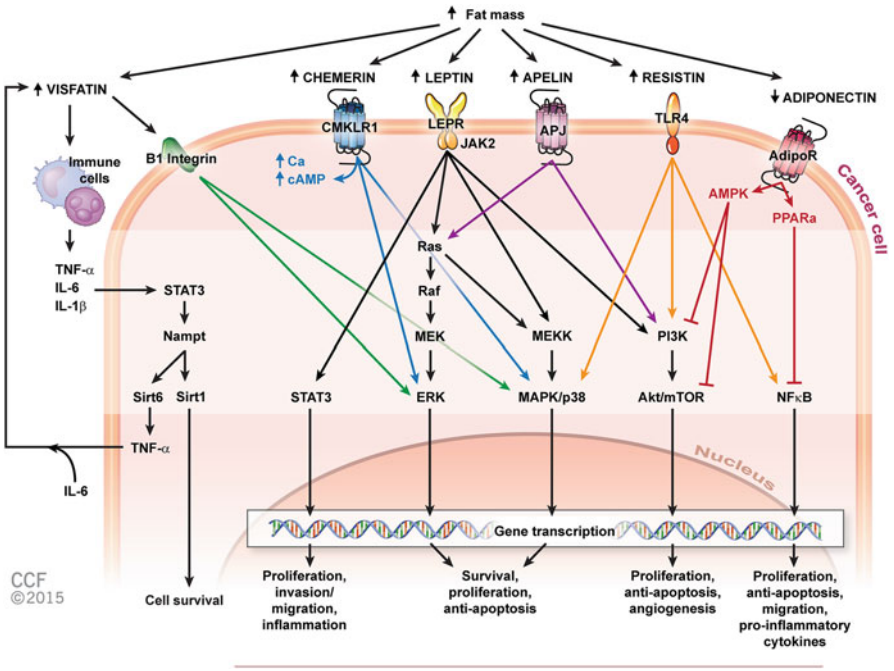

Fig. 1.1 Major adipokine signaling pathways promoting tumor growth and progression. Adipokines via their cell surface receptors activate major signaling hubs including Janus kinase (JAK)/signal transducer and activator of transcription (STAT), MAP/ERK, AMP kinase, and PI3K/AKT. These signaling pathways then lead to gene transcription activation that regulate cell proliferation, metabolism, survival, migration and invasion, angiogenesis, and tumor microenvironment. The major adipokines can activate complementary signaling and may be synergistic in activation of the common cell proliferation and survival effector pathways. Of note, Nampt is identical to visfatin, but here we refer to intracellular visfatin as Nampt to denote its nicotinamide phosphoribosyltransferase enzymatic activity

common malignancies observed among high-risk obese patients are cancers of the endometrium, colorectum, kidney, prostate, breast, and esophagus [14]. However, lesser degrees of association are also present for thyroid cancers, leukemia, non-Hodgkin's lymphoma, melanoma, and myeloma [15] (Fig. 1.2).

Based on epidemiological, pathophysiological, and mechanistic studies, adipocytokines constitute a major link between obesity and cancer [9]. Adipocytokines are implicated in carcinogenesis, tumor progression, recurrence, and metastasis. In addition to direct products of adipocytes, macrophages and other stromal cells play an important role in obesity-associated local inflammation which is particularly important for cancers arising and growing in fat-rich environments, including breast carcinoma, as well as for cancers that have a propensity to metastasize to fat-rich sites, such as gastric and ovarian cancers [2]. Adipocytokines can provide autocrine and paracrine signaling loops; however, they can also have systemic effects as they enter blood circulation to reach distant sites.

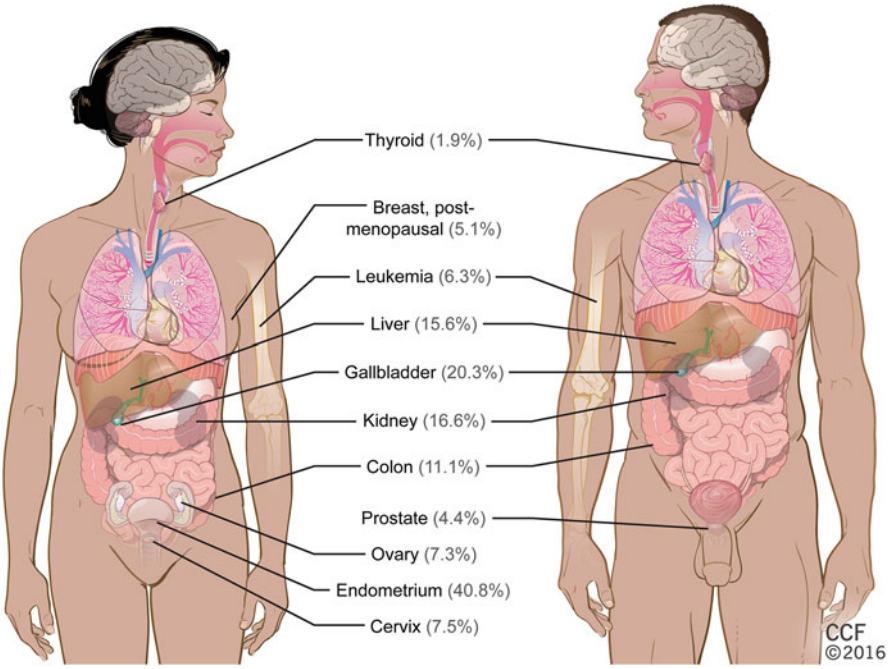

Fig. 1.2 Cancers associated with obesity. The schematic indicates the major organ site and associated increase in cancer incidence in obese patients. *Percentages* represent the cases attributable to being overweight and obese (BMI of 25 or above), combined for both genders [15]

Leptin

Leptin, also known as the "satiety hormone," is an adipokine, which was first identified in 1994 [12]. Leptin is primarily secreted from adipose cells within the adipose tissue though other tissues including the gut are able to secrete this cytokine. Of note, circulating leptin levels are proportional to body fat mass [16]. Leptin enters the circulation and originally was thought to interact solely with receptors in the brain. However, subsequent studies indicated that leptin has widespread targets including targets in muscle, liver, and adipose tissue [9]. Leptin is a cytokine, and the leptin receptor is a member of the GP130 family of cytokine receptors. There are six leptin receptor (LEPR) isoforms, also referred to as ObRa-ObRf, all encoded by a single gene [2]. Among these alternate splice isoforms, ObRb is of particular importance because it is the longest isoform and is mutated in the *db*/*db* mutant mouse [17]. The *db* mouse is a phenocopy of the *ob* mouse but is deficient in LEPR signaling [17]. LEPR is a single transmembrane protein that signals intracellularly via the Janus kinase 2 (JAK2)—signal transducer and activator of transcription 3 (STAT3) and

mitogen-activated protein kinase (MAPK) signal transduction pathways [17]. In the context of cancer, JAK2-induced activation of STAT3 and 5 induce transcription of genes involved in cell proliferation, invasion/migration, angiogenesis, and inflammation. SHP2 phosphorylation leads to activation of ERK/MAPK signaling pathway including activation of cell cycle inducers and inactivates tumor suppressor protein p53 [17].

Leptin is a cytokine secreted from the adipose tissue and elevated in obese individuals. Due to its role in regulation of body weight and link to obesity as well as its activation of mitogenic pathways, leptin has been an important focus of studies investigating the link between obesity and cancer [18]. Indeed, where studied, leptin has been shown to be mitogenic, antiapoptotic, pro-angiogenic, and pro-inflammatory and promote invasion and migration [9, 19]. It is implicated in cancers of the breast, colon, prostate, pancreas, ovary, and lung [9]. Multiple studies in breast cancer patients indicate that increased levels of circulating leptin is associated with higher risk of tumor progression suggesting it is an indicator of poor prognosis [19]. The growth-promoting effects of leptin are mediated through JAK/STAT, ERK, and PI3K signaling that ultimately lead to increased proliferation, cell survival, invasion and angiogenesis [9]. These cellular functions are the cardinal events taking place during oncogenesis. Moreover, in breast cancer models, leptin can inhibit apoptosis via upregulation of the bcl antiapoptotic genes (i.e., bcl-xL, bak, and bax) and induce angiogenesis through increased vascular endothelial growth factor (VEGF) production via stimulation of HIF-1α and NF-kB [16]. Furthermore, leptin causes an increase in aromatase activity and decrease in tumor suppressor protein p53 in MCF-7 cells which favors the survival of this estrogen receptor-positive breast cancer cell line [18]. In recent studies, leptin has been shown to increase survival of cancer stem cells in obese mouse models [20]. Subsequent studies indicated that leptin receptor is necessary for maintenance of cancer stem cells via regulation of the master regulators of stem cell self-renewal NANOG, SOX2, and OCT4 [21].

Similar to the association observed in breast cancer, patients with advanced prostate cancer have higher levels of circulating leptin compared to patients with benign prostate hyperplasia. Further, prostate cancer stage is correlated with circulating leptin levels, suggesting that leptin might be used as a biomarker for prostate cancer stage and prognosis [22].

In a cohort of patients with gastroesophageal adenocarcinoma, tumor leptin expression was associated with poor response to chemotherapy, a finding that supports the use of leptin as a biomarker of treatment responsiveness and a companion diagnostic [23]. In patients with gastric cancer, leptin was shown to increase invasiveness via Rho/ROCK signaling [24]. Leptin correlates with the aggressiveness of colorectal cancer [25], and its inhibitory effect on mitochondrial respiration has been linked to colorectal cancer progression [26]. Similarly, leptin promoted the growth of HepG2 liver cancer cells and inhibited apoptosis through blocking ER stress signals [27].

Adiponectin

Adiponectin is a protein hormone secreted exclusively from adipose tissue that regulates glucose homeostasis and fatty acid oxidation [9]. Circulating levels of adiponectin are inversely related to body fat stores and it is one of the few known adipokines with beneficial effects on health [28]. Adiponectin interacts with several receptors of which AdipoR1 and AdipoR2 are the best characterized [29]. These receptors are widely expressed in multiple tissues, including skeletal muscle, liver, vascular endothelium, hypothalamus, and white adipose tissue. Although AdipoR1 and R2 exhibit similarities to seven transmembrane domain receptors, they do not interact with G proteins, rather they activate the 5′-adenosine monophosphate-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor (PPAR)-α pathways. AMPK activation leads to increased energy expenditure and fatty acid oxidation, while increased expression of PPAR-α target genes (e.g., CD36, uncouplers, acyl-coenzyme oxidase) improves insulin sensitivity [11]. Moreover, activation of AMPK causes inhibition of PI3K/Akt pathway, and this effect causes a decrease in glycogen synthase kinase (GSK) activity and inhibits GLUT4 transport. These combined effects lead to decrease in cell growth and proliferation. Additionally, inhibition of mTOR causes loss of activation of S6K/eIF4E, which in turn leads to decrease in translation of cell cycle and angiogenesis genes [11].

Hypoadiponectinemia and decreased expression of AdipoR1 and AdipoR2 are seen in obesity and have been proposed as a potential link to diabetes, hypertension, atherosclerosis, and endothelial dysfunction [28]. In summary, adiponectin has opposing actions to leptin in cancer by being antiproliferative, pro-apoptotic, anti-inflammatory, and anti-migratory.

Reduced adiponectin levels are associated with cancers of the breast, colon, esophagus, liver, and endometrium [9]. Moreover, recombinant adiponectin has antitumor effects in myelomonocytic leukemia, breast adenocarcinoma, and fibrosarcoma [2]. Its anticarcinogenic effects have been demonstrated in different breast cancer cell lines, including MCF-7, MDA-MB-231, and T47D, in which adiponectin decreased cell proliferation and increased apoptosis [30]. Acting through AMPK phosphorylation, adiponectin decreased expression of cyclin D1 and c-myc, while increasing p53 and bax levels [31]. The anticancer effects are not limited to direct cellular activities as adiponectin leads to decreased growth factor bioavailability, including platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), and epidermal growth factor (EGF) [32].

Among other solid tumors, low levels of adiponectin predict an increased risk for prostate cancer [33]. High molecular weight form of adiponectin was shown to inhibit survival and proliferation of androgen-dependent and independent prostate cancer cell lines [34]. Similarly, several reports demonstrated a relationship between adiponectin and various GI cancers. Both increased leptin and decreased adiponectin, as seen in patients with obesity, are independent risk factors for progression of Barrett's esophagus to esophageal adenocarcinoma [35]. In addition, AdipoR1 and AdipoR2 were shown to be downregulated in gastric cancer as compared to normal gastric epithelium and thought to confer survival advantage for malignant cells [36]. An

inverse correlation was shown between adiponectin level and the number of colorectal adenomas, as well as the stage of colorectal cancer [37].

Decreased adiponectin expression is associated with high endometrial cancer risk in postmenopausal women. Adiponectin suppresses endometrial cancer growth via AdipoRs and increased expression of the adaptor molecule LKB1, which in turn modulates cell proliferation, colony formation, invasion, and adhesion [38]. Decreased AdipoR1 and AdipoR2 expressions are similarly also associated with higher histological grade, myometrial invasion, and lymph node metastasis [29]. Consistent with these findings, a recent study indicated that adiponectin levels were significantly lower in patients with endometrial cancer as compared to healthy controls [39].

Visfatin

Visfatin is secreted from adipose tissue, and blood visfatin levels positively correlate with body fat mass. While now recognized as a secreted protein, visfatin was originally defined as pre-B cell colony-enhancing factor (PBEF) [40]. Visfatin was also identified as nicotinamide phosphoribosyltransferase (Nampt), an enzyme catalyzing the rate-limiting step in NAD biosynthesis [41]. In 2002, Rongvaux, et al. showed that these PBEF and Nampt were the same gene [42]. Subsequent studies reidentified Nampt/PBEF as visfatin, which is secreted from visceral adipose tissue and initially proposed it to function in a similar manner to insulin, though this was subsequently rejected [43]. There is a positive correlation between visfatin and insulin resistance, metabolic syndrome, diabetes, and cardiovascular disease.

Visfatin may be secreted from neutrophils, monocytes, and macrophages as well [44]. Transcription of visfatin is regulated by pro-inflammatory cytokines, including TNF-α and IL-6, and its levels are increased in patients with inflammatory diseases [44]. Visfatin in turn increases the secretion of IL-1β, TNF, and IL-6 by immune cells via JNK and NF-kB pathways [45]. IL-6 increases the active Nampt in cells via STAT3 activation.

Visfatin has been implicated in cancer proliferation and invasion. Visfatin increases the activity of NAD-dependent enzymes sirtuin (Sirt) 1 and 6, which are associated with increased cell survival and TNF-α production, respectively [9]. A recent study by Wen-Shih Huang, et al. showed that visfatin can increase the expression of stromal cell derived factor-1 (SDF-1) in colorectal cancer via ERK and MAPK pathways which are activated through β1 integrin-mediated signaling [46]. This leads to increased cell survival and migration through chemokine receptors. In summary, visfatin is a pro-inflammatory cytokine, which can increase cell survival and migration and stimulates cytokine secretion.

Visfatin is associated with colon, breast, and ovarian cancers, as well as melanoma. It is closely linked to inflammation and enhances cell survival and SDF-1 expression. Colorectal cancer cells have chemokine receptors that can bind SDF-1, which in turn increases survival and migration of these cells [46]. Visfatin can also protect cancer cells from reactive oxygen species (ROS)-mediated damage via increased activity of superoxide dismutase, catalase, and glutathione peroxidase, an

effect that was shown in Me45 malignant melanoma cells [47]. Shackelford et al. have recently demonstrated that ovarian serous adenocarcinomas express significantly higher Nampt protein, which is through increased STAT3 signaling [48].

Resistin

Resistin is a small adipokine secreted mainly from mononuclear inflammatory cells, but also from adipose tissue in humans [9]. Therefore, it is highly linked to the inflammatory state and is thought to be an important link between obesity and inflammation [49]. The association between resistin and insulin resistance is less clear.

Resistin binds to Toll-like receptor 4 (TLR4) that in turn leads to the activation of PI3K, p38 MAPK, and NF-kB pathways. The principal effect of TLR4 activation is a surge in secretion of pro-inflammatory cytokines, which enhance the inflammatory state [50]. Similar to visfatin, activation of p38 MAPK leads to increased SDF-1 production, which underlies the observed cell migration. In addition, activation of PI3K and MAPK is associated with cell survival, proliferation, and inhibition of apoptosis. NF-kB is known to induce an increase in the expression of cell adhesion molecules, an upregulation of intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule-1 (VCAM-1) [51]. Collectively, the studies indicate that resistin is a pro-inflammatory adipokine that leads to increased cell proliferation, migration, and adhesion. Resistin is closely linked to inflammation and has been studied in several cancers including colorectal, gastric, breast, prostate, and liver tumors. TLR4 signaling and stimulation of PI3K pathway, together with NF-kB activation, can upregulate the synthesis of various cytokines which in turn causes a detrimental inflammatory response. Among the pro-inflammatory cytokines produced by these pathways, IL-6 has been shown to activate JAK/STAT and MAPK pathways and linked to epithelial-mesenchymal transition and metastasis of head and neck tumors [52]. Similar to visfatin, resistin induced expression of SDF-1 in gastric cancer cells through TLR4 signaling [53]. Recent studies highlight the role of resistin in increasing expression of ICAM-1 and VCAM-1 in Sk-Hep1 HCC cells, which is a cardinal step for tumor cell adhesion to endothelium. This carcinogenic effect was efficiently blocked by NF-kB inhibition [54]. In summary, tumorigenic effects of resistin include promotion of inflammatory milieu, immune cell extravasation, changes in expression of adhesion molecules, survival, and metastasis of cancer cells.

Apelin

Apelin is a recently identified peptide expressed in various tissues including liver, kidney, heart, lung, gastrointestinal tract, brain, adrenal gland, endothelium, and adipose tissue. The apelin receptor (APJ) is a G-protein-coupled receptor and acts via activation of ERK and PI3K/Akt pathways [55]. Apelin physiological functions include the

control of blood pressure, angiogenesis, cardiac tissue remodeling, hypothalamic regulation of food and fluid intake, and regulation of insulin and histamine release [56]. In normal-weight individuals, it has anti-obesogenic and insulin-like effects, but plasma levels are increased in obese patients as well as patients with type 2 diabetes [57]. Moreover, a recent study demonstrated that the pharmacological inhibitor of apelin (F13A) was promising in enhancing liver regeneration after hepatectomy [58].

High circulating levels of apelin, as observed with obesity, have been considered a risk factor for endometrial cancer [59]. Apelin was also suggested to have a role in lymphangiogenesis and lymph node metastasis [60]. In summary, apelin is pro-angiogenic, mitogenic, and can promote cell survival and migration.

Chemerin

Chemerin, also known as retinoic acid receptor responder protein 2 (RARRES2), is a small protein expressed in human lung, liver, and white adipose tissue [61]. It is a chemoattractant and acts via the G-protein-coupled receptor, CMKLR1, which is predominantly expressed on adipocytes and immune cells (e.g., neutrophils, macrophages, dendritic cells) [62]. Fat-derived chemerin has both autocrine and paracrine effects, and the amount of circulating chemerin secreted increases as the preadipocytes differentiate [61]. There is also a positive correlation between blood chemerin levels, BMI, and metabolic syndrome. CMKLR1-induced signaling includes both G-protein-coupled receptor-induced Ca^{2+} influx and cAMP increase and activation of MAPK and ERK pathways [9]. Increase in local chemerin concentration is associated with expansion of fat mass and local inflammatory response as evidenced by increased immune cell chemotaxis [62]. Therefore, chemerin contributes to the obesity-associated metabolic dysregulation and underlying chronic low-grade inflammation.

Chemerin is a pro-inflammatory cytokine of adipose tissue, which can enhance inflammatory response of tumor-infiltrating macrophages [35, 63]. However, data on the role of chemerin in tumorigenesis is limited, and the association has been best characterized for gastric cancer [64]. Serum chemerin concentrations correlated with the stage of gastric cancers and levels were higher in stage 1 patients as compared to healthy controls [64]. The mechanism by which chemerin induces invasiveness and metastasis in gastric cancers includes expression of VEGF, MMP-7, and IL-6 genes as well as activation of MAPK, ERK1/2, and p38 signaling pathways. It was also associated with poor postoperative prognosis and survival in gastric cancer [65].

Omentin

Omentin was originally recognized as intelectin-1, which is a lactoferrin receptor produced by intestinal Paneth cells and was thought to be involved in gut immunity [66]. Subsequent studies indicated that omentin is secreted from visceral adipose

tissue and enhances insulin-mediated glucose uptake in adipocytes via Akt signaling [67]. Omentin is implicated in promoting cell differentiation and inflammation via activation of AMPK/eNOS signaling and suppression of JNK activity, causing increased cell differentiation and dampened immune responses [68]. Other effects of omentin include vasodilation via eNOS activity and decreased expression of VCAM-1 and ICAM-1 leading to decreased monocyte adhesion via inhibition of ERK pathway. Blood levels of omentin are low in obesity, type 2 diabetes, and hypertension. Therefore, omentin is suggested to be a nutritional marker to reflect body weight and insulin resistance [69].

Omentin is an orphan adipocytokine, the role of which in carcinogenesis is not clear. There has been anecdotal reports linking increased serum omentin levels to prostate [70] and colorectal cancers [71], but its association with hepatocellular carcinoma is more pronounced [72]. It promotes apoptosis via upregulating p21 and increasing p53 and bax/bcl2 ratio [72].

Nesfatin

Nesfatin is an anorexigenic peptide, originally shown to regulate satiety through its action on hypothalamic nuclei [73]. Later studies demonstrated its expression in stomach, pancreas, testis, and adipose tissue as well. It modulates glucose homeostasis via AMPK and Akt signaling pathways and increases insulin sensitivity [74]. Nesfatin secretion is increased in obesity, and it might have a role in pathways leading to lipid accumulation [73]. A link between inflammation and nesfatin has also been suggested by the increase in its secretion in response to pro-inflammatory cytokines, including TNF-alpha and IL-6 [73]. Moreover, nesfatin-1 treatment inhibited cell proliferation in HO-8910 ovarian epithelial carcinoma cell line and promoted apoptosis in HO-9010 cells, which are due to effects on cell cycle and mTOR signaling, respectively [75].

Vaspin

Vaspin is a serine protease inhibitor produced mainly by the visceral adipose tissue, stomach, liver, pancreas, and the hypothalamus. Its levels are correlated with the amount of body fat stores and are linked to diabetes, obesity, metabolic syndrome, impaired insulin sensitivity, and coronary artery disease [76]. In rodent models, vaspin improves glycemia and reduces food intake [77]. Specifically, it interacts with GRP78 and activates Akt and AMPK, which in turn regulates glucose and lipid metabolism and improves metabolic dysfunction associated with obesity. However, underlying mechanisms of action associated with the beneficial effects of vaspin are not fully clarified.

Reports on the association between vaspin and cancer are very limited and controversial. The specific mechanisms of effect are also not fully clarified. It was found to be low in patients with endometrial cancer while higher in patients with colorectal cancer [9]. Therefore, at this point, it is hard to make a definitive statement on its role in tumor biology.

Retinol-Binding Protein-4

Retinol-binding protein (RBP)-4 is the carrier of retinol in the blood; it is mainly synthesized and delivered from the liver to peripheral tissues [9]. It is secreted also from adipose tissue and can act as a signal of low blood glucose. Several studies showed a positive correlation between RBP4 and insulin resistance or obesity, yet these studies have been hard to reproduce [11]. Due to its association with Glut4, RBP4 is thought to have a role in fuel sensing in adipocyte, though the mechanism underlying insulin resistance is not clear. Apart from its role in retinol transport, recent studies indicated that RBP4 can activate JAK/STAT signaling upon binding to its receptor, STRA6, and knockdown of the STRA6 receptor significantly inhibits growth of colon tumor cells in tissue culture and mice [78].

Cytokines

Expansion of fat mass, as seen in obesity, is associated with low-grade chronic inflammation that might in part be due to adipokines secreted from adipocytes (e.g., chemerin, visfatin). This leads to an increase in recruitment of macrophages into adipose tissue, exacerbating the inflammatory milieu through secretion of pro-inflammatory cytokines (e.g., IL-1B, TNF-α, IL-6, MCP-1). Moreover, obesity is associated with changes in the phenotype of macrophages, which causes them to convert into chronically active pro-inflammatory cells [11].

TNF-α is secreted from stromal macrophages and is known to disturb insulin signaling. Circulating TNF-α levels are higher in patients with insulin resistance [9]. TNF receptor signaling involves p38 MAPK, JNK, and NF-kB pathways, which can potentially culminate in transcription of genes involved in inflammation and cell survival. TNF-α also activates apoptotic pathways via activation of caspase cascade [11].

Similar to TNF-α, IL-6 is also increased in adipose tissue of obese individuals [11]. IL-6 impairs insulin signaling in adipocytes and hepatocytes through ubiquitination and degradation of insulin receptor substrate (IRS) [11]. IL-6 also functions via JAK/STAT signaling, and its significance in disease is a current area of intense research. Indeed, the IL-6 has been the focus of studies examining the link between obesity and cancer.

Adipocytes and Tumor Metabolism

As discussed earlier in the chapter, adipocytes are highly metabolically active, and adipocyte-derived factors affect cellular metabolism of malignant cells within the tumor. Fat cells promote growth, survival, and proliferation of malignant cells as they interact with them, an effect clearly shown in cancers of the breast, prostate, ovary, colon, and stomach [79]. An interesting study by Nieman et al. demonstrated that ovarian cells induce fat cell-driven lipolysis and increase lipid bioavailability for uptake by tumor cells in order to support the energy needs [80]. In parallel studies, prostate cancer cells have been shown to use beta-oxidation as a main source of energy [81].

Cancer cells undergo metabolic reprogramming during oncogenic transformation which leads to increased expression of glycolytic enzymes and in turn leads to high rates of glycolysis and lactate production independent from the presence of oxygen. Lactate provides carbon sources and sufficient energy for lipogenesis in rapidly dividing cancer cells [82]. Accumulation of fat in bone marrow has recently been linked to increased risk of development and progression of skeletal metastases, particularly in prostate cancer [83]. There are several hypotheses supporting this association, including adipocyte lipids supplying energy source for metastatic cells which induces proliferation, invasion, and motility and creation of an inflammatory milieu in the bone which favors tumor growth [2]. Therefore, in addition to secreting proteins, adipocytes generate metabolic products, including lipids and lactate, which may affect tumor growth. Moreover, adipocytes in bone marrow might mediate translocation of lipids to cancer cells. The complex interaction between lipid-driven and inflammatory pathways in the bone requires further investigation in order to fully clarify the role of marrow fat in metastasis.

Conclusion

Obesity is a systemic endocrine dysfunction with underlying chronic inflammatory state, rather than just an expansion of fat mass. Adipocytes promote growth of malignant cells via affecting and supporting their altered energy metabolism, particularly at sites where tumor cells are adjacent to fat tissue (e.g., skeletal metastases, ovarian and breast tumors). In addition, adipocytokines are cardinal mediators of carcinogenesis and tumor progression through their paracrine and endocrine effects. Single adipokines have independent roles in activating major intracellular signaling pathways involved in cell proliferation, growth, survival, adhesion, migration, and invasion. In addition, combined effects of adipokines should also be emphasized since they are dysregulated altogether in the setting of obesity.

Conflict of Interest None to declare.

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Chapter 2
Adiponectin, Obesity, and Cancer

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Abstract The worst outcomes of cancer, such as recurrence and mortality, are associated with obesity. Adiponectin is a fat-derived hormone and an important regulator of cell growth and tissue remodeling. Secreted exclusively from adipocytes into the peripheral blood, adiponectin contributes to tumor progression by acting locally and at distant sites. Contrary to expectations, adiponectin is decreased in obesity providing a link to tumorigenesis. Herein, we consider adiponectin's structure and post-translational modifications as critical determinants of its activity and receptor-binding ability. The cellular signaling pathways affected by the presence of adiponectin are discussed in the context of malignancy along with the leptin/adiponectin (L/A) ratio as an adjunctive tool in recurrence prediction. While the mechanisms involved in the paradoxical relationship between adiponectin, obesity, and cancer remain obscure, adiponectin replacement-based therapies may represent a novel preventative approach to diminish the incidence and mortality from obesity-associated cancers.

Keywords Adiponectin • HMW • Obesity • Cancer • AdipoR1 • AdipoR2 • Chemopreventatives

Introduction

Obesity is defined as abnormal fat accumulation in adipose tissue, which may lead to health impairment [1]. This rather inert definition masks the fact that the worst outcomes of cancer, such as recurrence and mortality, are in fact associated with obesity [1] (Fig. 2.1). The mechanistic links between adiposity and cancer

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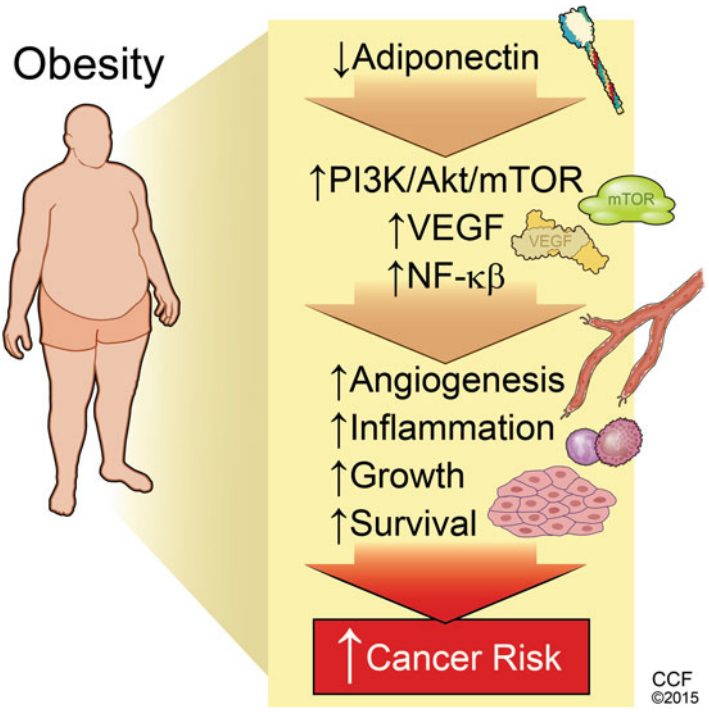

Fig. 2.1 Overview of the link between adiponectin, obesity, and cancer. Increased BMI (>30) is classified as an obese status. Circulating adiponectin levels in vivo are inversely correlated with the risk of malignancies associated with obesity. Decreased adiponectin activates members of the mTOR pathway (although inhibits mTOR), upregulates the PI3K/Akt signaling cascade, and increases phosphorylation of a range of molecules including NF-kB. Adiponectin mediates its activities via interactions with several important growth factors including VEGF. Reduced secretion from adipocytes into the circulation augments tumor angiogenesis, cellular inflammatory responses, and inhibits apoptosis leading to increased cancer risk

risk consist of (although are not limited to) insulin resistance, hyperinsulinemia, sustained hyperglycemia, oxidative stress [2], and of importance for this discussion—adipocytokines particularly adiponectin (30 Kda; APN, AdipoQ, ACDC, Acrp30, apM-1, APM1, GBP28). While these are all regarded as being responsible for cancer promoting effects, favoring tumor growth, increasing cell migration, and ultimately metastasis, it must be emphasized that contrary to expectations, adiponectin is found to be decreased in obesity [3]. Lower adiponectin levels in obesity are associated with chronic inflammation, endothelial dysfunction, enhanced oxidative stress, and insulin resistance [4].

Characterized in 1995 in differentiating mouse 3T3-L1 adipocytes [5], within a decade, the human homologue of adiponectin was identified as the most abundant transcript in adipose tissue. Recently, studies have shown that decreased plasma adiponectin (<4 μg/mL) is a key mediator in the development and possible progression of several types of obesity-associated cancers [6, 7], yet the mechanisms of this paradoxical relationship remain obscure. Nevertheless, a protective role for

adiponectin against several morbidities, including cancer, has been proposed. Understanding the central mechanisms linking adiponectin with cancer is expected to be of fundamental importance for the successful development and implementation of preventive and therapeutic strategies.

Adiponectin

Secreted exclusively from adipocytes into the peripheral blood, adiponectin circulates primarily as homo-multimeric full-length glycoprotein complexes [8] (Fig. 2.2). Adiponectin has a collagen-like motif and shares homologies with complement factors and TNF-α [5, 9]. Circulating adiponectin is secreted by adipose tissue in amounts inversely proportional to the body mass index (BMI) [10] and is directly related to visceral fat accumulation. Evidence linking adiponectin with insulin action stems from increased insulin sensitivity upon adiponectin administration with the muscle and the liver as target organs, resulting in an increase in skeletal muscle glucose uptake and hepatic fatty acid oxidation [11]. Recent evidence has shown that elevated adiponectin is correlated with enhanced β-cell function and fat loss after bariatric surgery [4]. This is supported by reports that reduced adiponectin expression occurs in parallel with the onset of insulin resistance in obese humans, rodents, and monkeys [9, 10, 12]. For the purposes of this discussion, the focus will be on adiponectin's role in obesity and its relationship with increased risk of malignancy.

Adiponectin Isoforms: Structural Considerations

Located on human chromosome 3q27.3, genomic sequence analysis determined that the adiponectin gene (ADIPOQ) spans 16 kb and contains three exons and a promoter lacking a TATA box [13–16]. Adiponectin comprises of 244 amino acids that represent a full 30 kDa protein [17]. It consists of four distinct regions: a signal sequence that targets the hormone for secretion outside the cell, a short region that varies between species, a 65 amino acid collagen-like motif, and a globular domain (Fig. 2.2). Structurally, adiponectin belongs to the complement 1q family and forms a characteristic homomultimer of various sizes. Intriguingly, when the three-dimensional structure of the globular region was determined, a striking similarity to TNFα was observed, despite unrelated protein sequences [18]. Human adiponectin can be present as a high (HMW), middle (MMW), and low (LMW) molecular weight form that corresponds to a multimer (as yet to be structurally characterized), hexamer (forms through self-association via the collagenous regions of two trimers), and trimer, respectively [19, 20]. Adiponectin trimers are generated when a triple helix is formed by non-covalent interactions between the collagenous regions and hydrophobic interactions between the globular head domains [18]. Adiponectin is highly abundant in human serum and circulates mainly as a 180 kDa MMW hexamer and a 360 kDa HMW multimer. A proteolytic cleavage product of adiponectin,

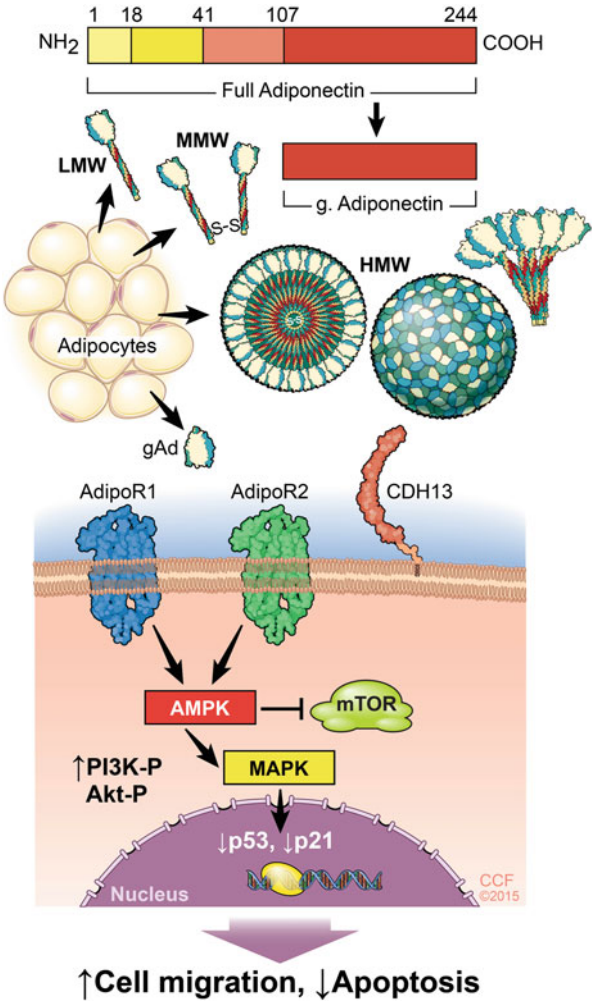

Fig. 2.2 Schematic of adiponectin and its signaling pathway in the context of malignancy. The full-length adiponectin (full adiponectin) contains in total 244 amino acids (aa) that represent a 30 kDa protein. It consists of four distinct regions: a short signal sequence (18 aa; *pale yellow*) that targets the hormone for secretion outside the cell, a short region that varies between species (22 aa; *yellow*), a collagen-like motif (65 aa; *brown*), and a globular domain (136 aa; *red*). Proteolytic cleavage releases the globular domain known as globular adiponectin (g. Adiponectin (gAd)). Secreted exclusively from adipocytes into the peripheral blood, human adiponectin can be present as high (HMW), middle (MMW), and low (LMW) molecular weight forms in the circulation. Three adiponectin receptors have been identified, two of which with homology to G protein-coupled receptors (adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2)) and one similar to the cadherin family (T-cadherin (CDH13)). The majority of adiponectin signaling in cancer is exerted via AMPK, which acts as an inhibitor of mTOR along with activation of MAPK. The proliferative effects of reduced adiponectin in tumors have been seen by upregulation via phosphorylation (P) of the PI3K/Akt signaling cascade. Reduced adiponectin directly promotes cell proliferation via decreased p53 and p21 expressions by inhibiting growth arrest and apoptosis in cancer

known as globular adiponectin (gAd), also circulates in human plasma [21]. HMW adiponectin is more rapidly metabolized than the trimeric form, but both forms are stable in vivo but do not interconvert.

Adiponectin circulates in human plasma in concentrations ranging from 3 to 30 μg/mL and contributes to 0.05 % of the total plasma protein [5]. When the production of adiponectin is reduced, either by obesity or in mice carrying only a single functional allele of the adiponectin locus, then the amount of the HMW form is selectively reduced in the circulation [22]. Recent clinical studies have revealed that the HMW complex is the most biologically potent form and plays a key role in the regulation of insulin resistance [23, 24]. There is a profound sexual dimorphism of adiponectin levels and complex distribution in serum. Females display significantly higher levels of HMW in serum than males with HMW forms becoming significantly reduced in response to a systemic increase of insulin [25]. We have previously reported that a comparison of multimer isoforms revealed a decreased percentage in MMW relative to HMW and LMW. The adiponectin SA ratio (HMW/total) was increased following exercise/diet interventions ($P < 0.05$) and correlated with the percent change in insulin sensitivity ($P < 0.03$) [26]. Recently, we also reported that seven consecutive days of aerobic exercise improve insulin sensitivity, fat oxidation, and HMW adiponectin in obese individuals independent of changes in body weight [27, 28]. New insights highlight the role of the relative distribution of adiponectin multimers as a more precise determinant governing adiponectin's defensive properties [8, 26, 29, 30]. Moreover, recent experimental evidence suggests that the antiapoptotic effect of adiponectin toward endothelial cells have been only observed with the HMW form, which specifically confers the vascular protective activities of this adipocytokine, [31] whereas globular adiponectin appears to exert an opposite effect [32].

Apart from its role as an anti-diabetic and anti-atherogenic hormone, adiponectin has been implicated as an important regulator of cell growth and tissue remodeling. It was shown that some of these functions might be mediated by the specific interactions of adiponectin with several important growth factors [33]. Among different growth factors examined, adiponectin was found to influence vascular endothelial growth factor (VEGF)-induced cellular migration and to bind with platelet-derived growth factor (PDGF), basic fibroblast growth factor (FGF), and heparin-binding epidermal growth factor (HB-EGF) with distinct affinities. The binding of adiponectin with these growth factors is oligomerization-dependent. PDGF binds to the high molecular weight (HMW) and middle molecular weight (MMW) complexes but not to the low molecular weight (LMW) complex. Basic FGF preferentially interacts with the HMW form, whereas HB-EGF binds to all three forms with comparable affinities. These three growth factors do not compete with each other for bindings to adiponectin, suggesting the involvement of distinct binding sites. The interaction of adiponectin with PDGF, basic FGF, and HB-EGF precluded binding to their respective membrane receptors and attenuated DNA synthesis and cell proliferation induced by them. Small interfering RNA-mediated downregulation of adiponectin receptors does not affect the suppressive effects of adiponectin on cell proliferation stimulated by these growth factors. These features collectively suggest that the

oligomeric complexes of adiponectin can modulate the biological actions of several growth factors by controlling their bioavailability at a pre-receptor level and that this effect might partly account for the anti-atherogenic, anti-angiogenic, and anti-proliferative functions of adiponectin [33]. Therefore, not only total concentrations but also multimer distribution should always be considered in the interpretation of plasma adiponectin levels in health as well as various disease states including cancer.

Adiponectin Receptors

Currently three adiponectin receptors have been identified, two of which have homology to G protein-coupled receptors (adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (AdipoR2)) and one that is similar to the cadherin family (T-cadherin (CDH13)) [34, 35] (Fig. 2.2). AdipoR1 and AdipoR2 have distinct affinities for the various circulating forms of adiponectin [36] and consist of seven transmembrane regions and internal N-terminal and external C-terminal domains. AdipoR1 is highly expressed in skeletal muscle but is present ubiquitously and binds globular and full-length forms of adiponectin with high and low affinity, respectively [35]. AdipoR2 is abundantly expressed in the liver and binds globular and full-length adiponectin with moderate affinity [37]. These two classical adiponectin receptors are structurally very related because their protein sequence shares 67 % identity and they are also highly conserved, sharing 95 % identity between humans and mice [38]. The third adiponectin receptor—CDH13—lacks a transmembrane domain and is located on the cellular surfaces of endothelial, epithelial, and smooth muscle cells. It binds to hexameric and HMW forms of adiponectin but not to trimeric or globular forms [34]. Several tumor cell lines express adiponectin receptors, suggesting that adiponectin could possibly exert direct effects on these cells by signaling through them. Adiponectin receptors are expressed in a plethora of malignant tissues including breast, prostate, hepatocellular, gastric, and colon carcinoma, pancreatic adenocarcinoma, and lung cancer [6, 39–43]. It should be noted that although almost every human tissue including cancerous cells can express various adiponectin receptors [44], one receptor usually prevails. The expression of AdipoR1/R2 is inversely correlated with plasma insulin levels in vivo under physiological (i.e., increase with fasting, decrease with feeding) and pathological conditions [45]. The protective role of adiponectin might be exerted either directly on potential cancer cells via receptor binding and by affecting signal pathways involved in cell growth [46] or indirectly by altering hormone and cytokine levels and therefore regulating whole-body insulin sensitivity [47]. We have previously shown that following an exercise/diet intervention, adiponectin receptor mRNA expression is significantly increased (AdipoR1 $P<0.03$, AdipoR2 $P<0.02$) and contributes in part to improved insulin sensitivity [26]. Although the functional relevance of adiponectin receptors in cancerous cells has not yet been clarified,

there is evidence that activation of adiponectin receptors limits the proliferation of cancer cell lines in vitro [6, 48, 49].

Adiponectin Modifications: Implication for Cancer

Adiponectin undergoes extensive and complex post-translational processing that is critical for the formation and secretion of adiponectin multimers. In humans, the most important modification is the hydroxylation and glycosylation of four conserved lysine residues (lys65, lys68, lys77, and lys101) and/or the hydroxylation of proline residues within the collagenous domain. Assembly of human adiponectin HMW oligomers depends on the disulfide bond formation mainly mediated by cysteine 39 [25]. These post-translational modifications are also crucial for the receptor-binding capabilities and biological actions of adiponectin [34, 50].

Adiponectin Signaling Pathways Related to Cancer

In the presence of malignant tissue, adipocytes can revert from mature, differentiated adipocytes to pre-adipocytes [51] and represent a major source of energy for the developing cancerous cell. Adiponectin is among the factors that contribute to tumor progression by acting on cancer cells as a local paracrine signaling cytokine. It can operate also at distant levels, through secretion from adipocytes into the circulation to modulate insulin sensitivity at the target tissue site, regulating inflammatory responses, and influencing tumor angiogenesis. Numerous cellular signaling pathways are affected by the presence of adiponectin. The majority of adiponectin signaling in cancer is exerted via the important cellular metabolic rate control point—5′ adenosine monophosphate-activated protein kinase (AMPK). Adiponectin directly controls cell proliferation, adhesion, invasion, and colony formation by regulating AMPK to promote growth arrest and apoptosis via increased p53 and p21 expressions, respectively [52, 53]. In breast cancer cell lines, elevated levels of adenosine monophosphate (AMP), calcium-dependent kinases, APPL-1, and LKB1 contribute to AMPK activation [54]. Adiponectin increases LKB1 expression in breast cancer cell lines, which results in AMPK activation and inhibition of tumor cell adhesion and migration [55]. AMPK interferes with cellular growth signaling by acting as an inhibitor of the mammalian target of rapamycin (mTOR), thus suppressing cell proliferation [56]. In the context of colorectal cancer, the treatment of cell lines with adiponectin resulted in suppression of the mTOR pathway, thus inhibiting cancer cell growth [57]. Other cellular signaling molecules influenced by adiponectin include phosphatidylinositol 3-kinases/protein kinase B (PI3K/Akt), mitogen-activated protein kinase (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), and members of the sphingolipid metabolic pathway. The proliferative effects of reduced adiponectin were seen in

MMTV-PyVT transgenic mice that developed mammary tumors by upregulating the PI3K/Akt signaling cascade [58]. Studies have shown that adiponectin treatment also reduces the phosphorylation of PI3K and Akt in breast and colorectal cancer cell lines [59]. The application of adiponectin on a hepatocellular carcinoma cell line resulted in increased c-Jun N-terminal kinases (JNK) activation and subsequent apoptosis via caspase-3 [60]. In vitro studies on endometrial and breast cancer cell lines showed that adiponectin prevented extracellular signal-regulated kinase (ERK1/2) signaling and resulted in decreased cellular viability [61, 62]. Moreover, treatment of MCF-7 breast cancer cell lines with adiponectin decreased c-myc, cyclin D, and Bcl-2 levels and increased Bax expression causing cell cycle arrest [62]. There is also evidence that adiponectin and T-cadherin interactions can influence tumor blood vessel growth and subsequent tumor aggressiveness [63], although the molecular mechanisms by which this interaction affects blood vessel growth has not yet been elucidated. Recently, a new mechanism was described whereby the balance between ceramide and sphingosine-1-phosphate (S1P) mediates many of the effects of adiponectin. AdipoR1 and AdipoR2 enhance ceramidase activity [64]. Many of the effects of adiponectin are mediated by ceramidase activity and the resulting alteration of the ratio of ceramide to S1P plays a role in cell growth [65].

Role of Adiponectin in Tumor Initiation and Progression

Adiponectin negatively influences growth of most obesity-associated tumors in both men and women. The obese setting provides a unique adipose tissue microenvironment with concomitant systemic endocrine alterations that favor both tumor initiation and progression. In adiponectin-deficient mice or wild-type mice fed either a high or low-fat diet, adiponectin supplementation minimized epithelial cell proliferation [66] and reduced implanted tumor growth [67].

Types of Cancer Associated with Obesity and Decreased Adiponectin

Playing a role not only in glucose and lipid metabolism as an insulin sensitizer, adiponectin also influences the development and progression of several obesity-related malignancies. The avoidance of weight gain in humans was shown to have a preventative effect for cancers of the colon, breast (post-menopausal), endometrium, kidney, and adenocarcinoma of the esophagus [68, 69]. Animal studies support these findings with evidence that caloric restriction dramatically decreases spontaneous and carcinogen-induced tumor incidence, multiplicity, and size [68, 69]. It has been suggested that circulating HMW adiponectin may be influenced by the magnitude of weight loss and correlate with body mass [70, 71]; however, we found no association between HMW adiponectin and weight loss suggesting that it is not the magnitude of weight loss but rather the location from which the fat is reduced or rather the change in fat

distribution that regulates adiponectin concentrations [26, 71, 72]. Circulating adiponectin levels in vivo were inversely correlated with the risk of malignancies associated with obesity such as colorectal cancer [73, 74], endometrial cancer [74], esophageal cancer [75], prostate cancer [76], breast cancer [77], gastric cancer [78], and hematological malignancies [79].

Colorectal Cancer

The pathological features (e.g., stage and grade) of colorectal tumor are affected by plasma concentrations of adiponectin [80]. The mechanism of tumor suppression by adiponectin is not fully understood although reports indicate that adiponectin can inhibit the expression of endothelial adhesion molecules, angiogenesis, and hematopoiesis [81]. Adiponectin also inhibits cell growth and induces apoptosis in a dose-dependent manner, both in vivo and in vitro [82]. A case–control study nested in the large prospective health professionals' follow-up evaluation found that low-plasma adiponectin levels are associated with risk for colorectal cancer in men [69]. Individuals in the highest adiponectin quintile have an approximately 60 % reduced risk for colorectal cancer compared to the lowest quintile, the association being independent of body mass index, waist circumference, waist-to-hip ratio, and physical activity [46]. Decreased concentrations of plasma adiponectin are associated with the development of colon adenomas in Japanese patients [83], the association being particularly significant with the number/size of tumors and histological progression from tubular to tubulovillous/villous adenomas. Furthermore, several case–control studies [83–85] have confirmed the occurrence of lower adiponectin levels in patients with colorectal cancer than in healthy controls, and it was recently suggested that adiponectin might represent a prognostic parameter in risk prediction for its recurrence [83, 86]. Exogenous administration of adiponectin in an *Apc*Min/+ mouse model of intestinal carcinogenesis was shown to be capable of reducing the growth of intestinal polyps [87]. It was also reported that adiponectin knockout mice are more prone to chronic inflammation-induced colon cancer compared to wild-type mice [88, 89] with adiponectin-modulating genes involved in chronic inflammation and tumorigenesis [89]. Recent studies exploring HMW and non-HMW adiponectin fractions in relation to colorectal risk showed that, when stratified by cancer site, non-HMW adiponectin is inversely associated with both colon and rectal cancer, suggesting an important role of the relative proportion of non-HMW adiponectin in colorectal pathogenesis [90].

Endometrial Cancer

Adiponectin was reported to be inversely related with risk of endometrial cancer, especially among women < 65 years [74]. Interestingly, in women diagnosed with endometrial cancer, obesity was more prevalent in pre-menopausal, compared

with post-menopausal women [69]. The combination of high BMI and low adiponectin levels are associated with a more than six fold excess risk of endometrial cancer [74, 91, 92], and in a recent meta-analysis, it was concluded that a high serum adiponectin concentration was associated with a reduced risk of developing endometrial cancer, particularly in post-menopausal women not taking hormone replacement therapy [93].

Prostate Cancer

In a case–control study, plasma adiponectin levels were reported to be significantly lower in subjects with prostate cancer than in those with benign prostatic hyperplasia or in normal healthy controls [76]. This is supported by findings showing that plasma adiponectin levels inversely correlate with histological grade and disease stage [76].

Breast Cancer

Obesity increases rates of breast cancer by 30–50 % in post-menopausal women [69]. Because adipose tissue cells represent the predominant breast stromal element, adiponectin applies a major paracrine influence in mammary epithelium. It is believed that adiponectin may play a role in breast cancer etiopathogenesis, particularly in the low-estrogen environment observed in post-menopausal women. Additionally, it has been suggested that breast tumors arising in women with hypoadiponectinemia present a more aggressive phenotype in the context of larger size of tumor, higher histological grade, and estrogen receptor negativity [91, 92, 94]. Observations based on a case–control study and confirmed by subsequent studies have shown that lower circulating adiponectin levels are associated with increased risk of breast cancer [91, 92, 94] independent of age, menopause status, hormone receptor status, lymph nodes metastases, and status of human estrogen receptor (ER) [94]. Although a significant reduction in tumor volume was observed recently in animals injected with ER-α-negative MDA-MB-231 cells pre-treated with adiponectin [95]. It has been shown that AdipoR1/R2 are expressed in breast cancer cell lines and tissue samples and that adiponectin may act not only via altering the hormonal milieu but directly through inhibition of breast cancer cell proliferation in vitro [49]. Jeong et al. [96] found that high adiponectin and AdipoR expression may also be associated with breast cancer invasiveness.

Gastric Cancer

Lower serum adiponectin levels have been reported in patients with gastric cancer especially with upper gastric cancer compared to healthy controls [78]. Moreover, the adiponectin concentration appears to be reduced as the tumor stage increases although it should be noted that BMI does not differentiate the stages [97].

Hematological Malignancies

In the bone marrow milieu, adiponectin and its receptors are expressed by the majority of bone marrow stromal cell populations influencing hematopoietic stem cell function. Leukemia, multiple myeloma, myeloproliferative disorders, Hodgkin's and non-Hodgkin's lymphoma, and myelodysplastic syndromes are linked to the role of the bone marrow microenvironment and hypoadiponectinemia. Thus, adiponectin may represent a molecular mediator linking adiposopathy with leukemogenesis and myelomagenesis [79].

The L/A Ratio

Leptin is a fat-derived hormone that is intricately linked to obesity. Excess body fat is associated with increased expression of leptin and downregulation of adiponectin [98]. Overall, studies suggest that leptin plays a role in tumor development and progression, whereas adiponectin plays a role in tumor inhibition [99]. In one prostate cancer model, adiponectin reduced cell proliferation, and this effect was blocked by treatment with leptin [100]. Thus, leptin and adiponectin have been suggested to have opposing roles in cancer development and progression. Meanwhile, evidence from a large prospective study evaluating the association of plasma adiponectin and soluble leptin receptor (sObR) with colorectal cancer risk demonstrated that plasma adiponectin was significantly associated with reduced risk of colorectal cancer among men, but not among women, and that sObR was significantly associated with increased risk of rectal cancer but not colon cancer [101]. Recently, the hypothesis has been proposed that an unfavorable adipokine profile [as indicated by a high leptin/adiponectin (L/A) ratio] might serve as a prognostic factor in colorectal cancer patients [86, 102]. In particular, the evidence that a high L/A ratio has a negative prognostic value (independent of gender) with respect to both disease-free and overall survival in colorectal cancer patients suggests that combined measurement of both adipokines may represent an adjunctive tool in risk prediction for malignancy recurrence [86].

Adiponectin Chemopreventatives

Recent research indicates that adiponectin or analogs might be useful agents in the management or chemoprevention of obesity-related cancer. For example, adiponectin replacement-based therapies may represent a novel approach to prevent growth of early stage colorectal cancer. A recent report described the first orally active adiponectin receptor activator AdipoRon—an adiponectin-like synthetic small molecule—that activates the adiponectin receptors AdipoR1 and AdipoR2 [82], mimics the anti-proliferative effects of adiponectin, and is suggested to perhaps act as a colorectal cancer suppressor. Data on AdipoRon were reported for effects on insulin resistance, type 2 diabetes, and longevity in an obese diabetic mouse model. The effects were similar to adiponectin and worked via AMPK pathway activation with induction of AMPK phosphorylation and peroxisome proliferator-activated receptor alpha (PPAR-α) activation in muscle and the liver. Survey results demonstrated improvements in insulin resistance and glucose intolerance and, also, lower plasma glucose, increase in fatty acid oxidation, oxidative stress reduction, increased life expectancy, and a decrease in expression of pro-inflammatory cytokine coding genes such as tumor necrosis factor alpha (TNF-α). A follow-up mouse study using AdipoRon treatment against myocardial ischemia/reperfusion-induced apoptotic cell death reported evidence for significant improvement in adiponectin knockout (APNKO) mice [103]. No study to date has examined the effect of AdipoRon on colorectal cancer proliferation. Further investigation into the role of such chemopreventative agents is warranted to determine their role against risk of developing malignancy.

Concluding Remarks

Adipose tissue is now regarded as not just a storage reservoir for excess energy but rather as an endocrine organ, secreting bioactive molecules including adiponectin. Increased body weight and accumulation of central adiposity lead to reduction in adiponectin levels, decreased insulin sensitivity, and the complications of obesity [104]. Reversing obesity-associated inflammation and adiposopathy by lifestyle interventions, such as physical activity and dietary modifications, might have a clinically relevant role in reducing cancer risk or progression, and influence survival outcomes. Mechanistic and pathophysiological studies on adiponectin highlight the important role of this fat-derived hormone in cancer. Dissecting the mechanisms underlying adiponectin's involvement in obesity-driven malignancy will be of utmost importance for risk reduction and design of tailored therapies to prevent chemo-resistance and recurrence. The exponential growth of obesity and associated cancers presses the need for effective therapeutic interventions that combine lifestyle changes with adiponectin-based analogs to promote the preventative effects of decreased adiposity and diminish the incidence and mortality from cancer to improve the life trajectory of modern society.

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Chapter 3
Leptin in Cancer: Epidemiology and Mechanisms

Margot P. Cleary and Marta Torroella-Kouri

Abstract Obesity is now considered a risk factor for many cancers. In particular, the risk of developing postmenopausal breast cancer is elevated in overweight/obese women. Leptin, an adipokine made in adipose tissue, may be a physiological link between cancer and obesity. Epidemiological studies in some cases report elevated leptin levels in women with breast cancer and expression of the leptin receptor (Ob-R) in breast tumors. In obese transgenic mice predisposed to breast cancer that were leptin or Ob-R deficient, no mammary tumors developed, while in similar transgenic mice with diet-induced obesity, mammary tumor latency was shortened, and leptin levels were correlated with body weight. In vitro studies consistently report leptin enhances in cell proliferation and cell signaling pathways such as STAT3/AKT/PI3K. Leptin has also been recently reported to enhance angiogenesis through VEGF and its receptor, VEGFR. Other recent studies show that leptin may promote inflammation in tumor cells as well as nearby stromal components and adipocytes. Further leptin may have effects on cancer stem cell populations promoting early stages of tumorigenesis. Leptin has also been shown to play roles in the development of hepatic, colorectal, ovarian, and endometrial cancers and in the development of aggressive prostate cancer. The development of Ob-R agonists and leptin analogues may help treat and/or prevent some cancers.

Keywords Leptin • Leptin receptor • Breast cancer • Obesity • Adipocytes • Mice • Stem cells • Agonists • Cancer

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Over the past 20 years, there has been increasing interest in the role of obesity as a risk factor associated with the development and/or progression of many types of cancer. Breast cancer has been of particular interest with numerous review articles addressing this relationship. Meta-analyses of published experimental studies have indicated that obesity, especially that resulting from substantial weight gain in middle aged, increases a postmenopausal woman's risk of developing estrogen receptor (ER)-positive (+) breast tumors [1–3]. This relationship may be tempered by whether women have used hormone replacement therapy [2]. In addition, it appears that regardless of menopausal status, i.e., either pre- or postmenopausal at the time of diagnosis or tumor characteristics (ER+/ER− and/or HER2 positive/negative), overweight/obesity is associated with a worse prognosis including tumor recurrence and death [4, 5].

These observations have led to the question, "What is/are the factor(s) associated with increased body weight that are involved in this process?" Initially, the major focus was on the conversion of androgens to estrogens by enhanced aromatase activity in the expanded adipose depots of obese women as reviewed [6]. However, the identification of proteins produced in exclusively or in large amounts of adipose tissues subsequently termed adipokines has considerably expanded the possible players in this relationship. These potential growth factors would provide a direct association of obesity to breast cancer through the circulation. Further, given the presence of adipose tissue in the breast itself, this would be a potential local source of these growth factors. Thus, there has been an increasing interest not only in systemic factors but the microenvironment and paracrine effects that could contribute to increased proliferation and altered cell signaling resulting in the tumorigenesis process related to adipose depots and/or body weight status.

Here we will focus on leptin which was identified in 1994 as the missing factor responsible for the excessive body fat in genetically obese *ob*/*ob* mouse (subsequently renamed $Lep^{ob}Lep^{ob}$) [7]. Initial studies indicated that leptin interactions with its receptor, Ob-R, in the hypothalamus controlled satiety and monitored body fat stores. However, as investigations progressed Ob-R was identified in numerous other tissues throughout the body and eventually in many tumor types as well as in cancer cell lines indicating that leptin signaling had more extensive physiological actions. Also it was noted that serum/plasma levels of leptin were increased as body weight increased [8, 9]. This protein subsequently was considered the first of many adipose-derived proteins now termed adipokines. Further, the fact that leptin was made in adipose tissue provided a potential direct link of obesity to the development and progression of cancer.

An Overview of the Early Studies of Leptin and Breast Cancer

In 2002 three publications reported that Ob-R was present in various human breast cancer cell lines and that the in vitro addition of leptin increased cell proliferation [10–12]. Numerous signaling pathways were also identified to be associated with leptin's ability to enhance proliferation (including JAK/STAT, ERK1/2, AKT-GSK3, and PKC-3) [11, 13–15].

In parallel with these, in vitro study attempts were made to determine if serum leptin levels were involved with the development/diagnosis of breast cancer. However, as previously reviewed [16] results were inconsistent. This was possibly due at least in part to studies not being designed for this purpose, combining pre- and postmenopausal subjects, using only premenopausal women and timing of obtaining the samples during the disease process. Additional studies evaluated leptin and/or Ob-R status/levels in breast tumors. It was reported that both proteins were overexpressed in breast tumors [17] and also that high expression of Ob-R was associated with poor prognosis in relation to high-serum leptin levels [18].

There have been previous reviews addressing the potential role of leptin on breast cancer [16, 19–25]. Therefore, here we will present an update focusing on publications primarily from the past 5–6 years. We will include recent studies related to leptin and breast cancer, in vivo rodent studies and in vitro publications. We will also discuss areas of recent interest such as cancer stem cells, tumor microenvironment, and analog/antagonist studies and will present highlights related to leptin in other cancers.

Epidemiological Studies of Leptin and Breast Cancer

There have been several recent epidemiological publications on the topic of leptin's association with breast cancer. In one cross-sectional study of Mexican women, newly diagnosed obese nondiabetic breast cancer patients, with BMI > 30, were compared to obese women without breast cancer at their annual checkup [26]. Women with breast cancer had higher-serum glucose, leptin and HOMA-IR, and reduced estradiol compared to the cancer-free obese controls. The height and weight of the women were determined by health personnel which is a plus for the study; however, the control women were significantly younger (46.1 vs. 53.0 years $p < 0.05$), and parity was also different between controls and women with breast cancer. In addition results for pre- and postmenopausal subjects were combined. There were a total of 76 women in each of the two groups.

A case-control study of Saudi Arabian women also newly diagnosed with breast cancer and BMI (average ~31) matched to control subjects which reported higher-serum leptin and reduced adiponectin concentrations in women with breast cancer [27]. Other significant findings included higher TNF-α, glucose, C-reactive protein (CRP), and triglycerides but lower HDL cholesterol in the breast cancer subjects. However, there was a significant difference in the proportion of women who were menopausal with the breast cancer subjects having 40 % (22/56) compared to only 17 % (9/53). It should be noted that the focus of this study was on metabolic syndrome biomarkers.

Analysis of blood samples obtained from postmenopausal women in the Multiethnic Cohort Study 3–8 years before breast cancer diagnosis indicated that those with breast cancer ($n = 706$) compared to controls without breast cancer ($n = 706$) had significantly higher leptin, leptin/adiponectin ratio, and CRP which

were independent of BMI [28]. There were however no differences in serum adiponectin levels. Later in this chapter, we will discuss in more detail the potential usefulness/impact of the leptin/adiponectin ratio in breast cancer.

In addition to serum measurements of leptin there have been analyses of leptin and/or its receptor in tissue samples. For example, Gnerlich et al. [29] conducted a pilot study ($n=19$) using stromal tissue obtained near, as well as distant from the breast tumor in women with average BMI of 28.1. Leptin expression (immunohistochemistry) was higher in the tissue near the tumor as compared to that which was distal in obese (BMI > 30) women. The focus of the study was breast adipose tissue and the protein pigment epithelium-derived factor and adipose triglyceride lipase and not leptin per se.

A nested case-control study [30] focused on premenopausal breast cancer using information obtained from subjects in the Nurses' Health Study II. Blood samples obtained prior to cancer diagnosis were analyzed for polymorphisms of leptin and Ob-R as well as for leptin serum concentrations. Subjects were primarily Caucasian, and women with and without breast cancer were matched for many factors and in this case also for luteal phase. There were no significant differences obtained for the breast cancer ($n=405$) or control subjects ($n=810$) with respect to serum leptin or the association of SNPs for leptin or its receptor.

A number of additional studies have tried to determine if leptin/Ob-R polymorphisms are associated with breast cancer. In an early study, Snoussi et al. measured polymorphisms of leptin and Ob-R in Tunisian women with breast cancer ($n=308$) in comparison to controls ($n=222$) [31]. Breast cancer risk was significantly associated with heterozygous leptin (−2548) GA and homozygous leptin (−2548) AA variants as well as with heterozygous Ob-R 223QR or homozygous Ob-R 223RR genotypes. Decreased disease-free survival was significantly associated with the presence of the leptin (−2548) A allele, whereas the presence of Ob-R 223R had a significant association with decreased overall survival. This polymorphism for leptin (G-2548A) was also reported to be associated with breast cancer in a recent case-control study in Iran where the average age of breast cancer subjects was 44.8 ($n=203$) vs. 42.2 years for controls ($n=171$), and the other genotypes were associated with a reduced risk [32]. The average BMI for women in this study was approximately 26.

Several meta-analyses on the topic of polymorphisms have been recently published. A meta-analysis of nine studies suggested some, i.e., two of five Ob-R polymorphisms may be related to the development of breast cancer [33]. A second meta-analysis of 17 studies was performed to assess the associations of five polymorphisms on the leptin, OB-R, and PON1 (paraoxonase 1) genes (LEP G2548A, LEPR Q223R, LEPR Lys109Arg, PON1 L55M, and PON1 Q192R) with breast cancer risk. The studies produced mostly negative results except for PON1 L55M, which was significantly associated with breast cancer risk overall and for LEPR Q223R polymorphism which might be implicated in the development of breast cancer in East Asians [34].

Tumor and non-tumor samples obtained from British women have also been analyzed for leptin and Ob-R mRNA [35]. Leptin and Ob-R expressions were higher in tumors than non-tumor tissue with no effect of tumor grade or ER status. In addition

leptin and Ob-R levels were correlated. In another study samples from Chinese women were used for measurement of leptin and Ob-R expression in tumors and stroma [36]. For leptin a significant increase in its expression in the cytoplasm of triple-negative tumors was reported in comparison to either ER− or ER+ tumors. Leptin and OB-R were detected in more than 61 % of samples and leptin positively correlated to OB-R, JAG1, VEGF, and marginally to IL-1R.

In a recent study, microdialysis of breast tumors and abdominal subcutaneous adipose tissue from postmenopausal women ($n = 18$) with ER+ tumors was undertaken. Samples were obtained before and after 6 weeks of tamoxifen treatment which significantly reduced leptin levels in the breast, while adiponectin increased resulting in a decrease in the leptin/adiponectin ratio. However, these two adipokines were not affected by tamoxifen treatment in subcutaneous adipose tissue [37]. Other aspects of this study included culture of breast tissue with or without tamoxifen, but no effects on adipokines or their receptors were found. Also normal breast tissue from premenopausal women exhibited differences in adipokines dependent upon the phase of menstrual cycle and exposure to different levels of estradiol, but no such changes were obtained in abdominal subcutaneous adipose tissue.

Recent In Vitro Studies

As indicated in the Introduction, a number of in vitro studies analyzed breast cancer cell lines and verified that the Ob-R was present and the addition of leptin enhanced cell proliferation and impacted multiple signaling pathways. In general results indicated that leptin had a more robust effect on cells expressing ERα. Overall these findings provided evidence that leptin had the potential to be a growth factor related to the presence of obesity. Over the past 5–6 years, additional publications have further supported this action of leptin addressing various consequences of how leptin and/or its receptor may play a role in the development of breast cancer. Highlights of these studies will now be presented.

Binai et al. have several recent publications [38, 39] which further support that the presence of ERα is important for leptin-induced activation of STAT3. The first study included comparisons of triple-negative MDA-MB-231 cells with ER+ MCF-7 and the use of techniques to interfere with ERα signaling [38]. In the second study, MDA-MB-231 cells were transfected with Ob-RL (the long signaling form of Ob-R) or ERα [39]. These cell lines were used to identify genes regulated by leptin-affected ER expression which could contribute to progression of breast cancer. Another publication with focus on ER status [40] compared the response to leptin on CYP1B1 in ER+ MCF-7 with triple-negative MDA-MB-231 breast cancer cell lines. Leptin appeared to induce CYP1B1 through ERα by way of AKT and ERK activation. These two cell lines were also used to assess interactions of leptin and its receptor with ERα [41]. Different responses to leptin (100 ng/ml) on Ob-R expression were evaluated. There was an increase in Ob-R expression in MCF-7 but none in MDA-MB-231 cells. Further, STAT3 and ERK1/2 were phosphorylated by the

addition of leptin in MCF-7 cells, but there was no effect on the already high phosphorylation levels in MDA-MB-231 cells. Further leptin caused a dose-dependent increase in proliferation in MCF-7 cells—but had no effect in MDA-MB-231 cells. The addition of anti-OB-R neutralizing mAb and 9F8-blocked leptin effects in MCF-7 but not in MDA-MB-231 cells and silencing of the OB-R gene only affected MCF-7 cells. In breast tumors a high expression of Ob-R was associated with ERα expression.

Dubois et al. [42] used multiple breast cancer cell lines, i.e., MCF-7, T47D, and MDA-MB-231 as well as MCF10A cells (fibrocystic breast tissue) and 184B5 cells (normal breast tissue) to study the relation of leptin in breast cancer. They identified leptin and its receptor mRNA and protein in all five cell lines. The addition of leptin at either physiological (10 ng/ml), obese (100 ng/ml), or pharmacological (1000 ng/ml) concentrations resulted in leptin-stimulating proliferation of all cell lines except for 184B5 and MDA-MB-231 cells. It should be noted that these studies were done with and without fetal calf serum which impacted some of the effects of leptin. Apoptosis and cell cycle were assessed only in cells responsive to leptin in the proliferation study, and it was found that leptin decreased apoptosis in MCF-7 cells. Yan et al. [43] published a recent paper on leptin induction of EMT transition using primarily MCF-7 cells. The focus was on leptin's effects on Wnt1/β-catenin pathway. In a study using the mouse-derived mammary tumor, 4T1 cells increasing concentrations of leptin enhanced IL-6 production [44].

Most cell culture studies are fairly short term, i.e., 48–72 h. However, Nadal-Serrano et al. [45] recently published the results of a long-term, 10-day cell culture of MCF-7 cells. The cells were cultured with leptin (100 ng/ml) which increased proliferation by 30 % as well as production of ROS and SIRT1, while 4-HNE adducts and carbonyl groups were decreased.

Terrasi et al. [46] examined five cell lines for Lep-2548G/A polymorphism in the leptin promoter region that might affect leptin secretion. Homozygous (A/A) gene expression was found for this polymorphism in BT-474 and SK-BR-3 cell lines, heterozygous formation in MDA-MB-231 cells, and wild-type G/G in MCF-7 and ZR-75-1 cells. Both homozygous and heterozygous gene expressions were associated with high- and intermediate-leptin mRNA expression activating through Sp1 and nucleolin-dependent mechanisms.

Co-culture systems have been used recently to evaluate cross talk between different components of the breast that contribute to tumorigenesis. With respect to leptin, Barone et al. [47] used both wild-type and K303R mutant overexpressing ERα breast cancer cells grown with cancer-associated fibroblasts (CAF). Ob-R isoform and activation of leptin-signaling pathways were increased in the mutant cells. Conclusions from this study included that there is a two-way communication between stromal and cancer cells that through leptin provides an explanation for obesity's role in breast cancer development. Another recently published co-culture experiment used either MCF-7 (ER+) or triple-negative MDA-MB-231 breast cancer cell lines cultured with adipose stem cells from either obese or lean human subjects [48]. Increased cell proliferation occurred in MCF-7 cells co-cultured with obese adipose stem cells obtained from either subcutaneous abdominal or non-abdominal subcutaneous

adipose depots of obese and lean subjects, but the abdominal cells resulted in the highest proliferation. In contrast, although co-culture increased MDA-MD-231 cell proliferation, the increase was similar for stem cells obtained from either lean or obese subjects and regardless of site. With respect to leptin, abdominal stem cells from obese subjects demonstrated increased expression of leptin and further estrogen-stimulated leptin expression in the stem cells which then enhanced MCF-7 cell proliferation. Further the addition of leptin neutralizing antibody with estrogen prohibited the estrogen-stimulated increase in proliferation. This led to the conclusion that this effect on cell proliferation in MCF-7 cells is mediated through estrogen activation of leptin.

Preclinical Rodent Studies Evaluating Leptin's Effects on Mammary Tumor Development/Progression

Rodent studies have been utilized to clarify leptin's role in mammary tumor development and progression. Several early studies were designed to assess the effect of genetic obesity on the development of mammary tumors independent of diet. Unknown at that time was that the two genetically obese mice strains were obese due to defects in leptin signaling. In the first study [49], transgenic MMTV-TGF-α [50] mice that develop mammary tumors slowly into the second year of life [51] were crossbred with $Lep^{ob}Lep^{ob}$ mice, and the second $Lepr^{db}Lepr^{db}$ mice were used [52]. As it turned out, these two strains were leptin deficient [7] or Ob-R deficient [53]. Following the crossbreeding mice were followed for two years to determine mammary tumor latency and incidence. In both situations mammary tumors did not develop in the homozygous obese mice in contrast to their wild-type or heterozygous siblings that developed mammary tumors as expected. Although these findings provided strong support for a role of leptin in tumorigenesis, the fact that the obese mice also had problems with mammary tissue development confounded the issue.

More recently these two mouse strains have been used in a short-term study of mammary tumor progression using a syngeneic mammary tumor cell line developed from MMTV-Wnt-1 mice [54]. Tumor volume and growth rate were much greater in the obese $Lepr^{db}Lepr^{db}$ mice that had high circulating levels of leptin (24.4 ng/ml) in comparison to the equally obese $Lep^{ob}Lep^{ob}$ mice in whom leptin was not detected or to normal-weight wild-type mice with normal circulating leptin (0.9 ng.ml). Adiponectin levels were not significantly different among the groups although they were about 25 % and 33 % lower in $Lep^{ob}Lep^{ob}$ and $Lepr^{db}Lepr^{db}$, respectively, compared to normal-weight mice. In another study using this cell line, Ob-R-silencing reduced tumor formation compared to the non-silenced cells [55].

Tumor progression in relation to leptin and aromatase was investigated using a xenograft model with MCF-7 cells [56]. The cells were inoculated with or without F442A adipocytes, and the presence of the adipocytes clearly enhanced tumor growth. Leptin was also injected near the F442A cells and aromatase gene

expression determined 3 h later. Leptin treatment increased aromatase gene expression sixfold. Also there was an increase in fat pad aromatase mRNA following consumption of a high-fat diet, while in *Lep*^{ob}*Lep*^{ob} mice, aromatase was lower compared to lean mice.

An additional study supporting a role of leptin in tumor progression indicated that reduced serum leptin levels were associated with reduced mammary tumor growth following implantation of the EO771 mouse mammary tumor cell line in diet-induced obese mice with hypothalamic gene transfer of BDNF (brain-derived neurotrophic factor). Other changes included lower body and fat weights but no change in serum adiponectin, IGF-I, insulin, and glucose [57]. Morad et al. [37] used MCF-7 cell xenografts and middle PyMT-derived tumor allografts where microdialysis was done to assess estradiol effects on leptin and adiponectin at the tissue level. No local extracellular levels of leptin and adiponectin were detected by microdialysis or examination of tissues by immunohistochemistry. FVB/N mice with MMTV-PyMT tumors in the presence of estradiol had higher levels of leptin and leptin/adiponectin ratio compared to tumors in estrogen-depleted mice. Some decrease in adiponectin was found but was not significant due to the small number of animals used ($n=7$). Interestingly, Ob-R staining was more intense in tumors grown in the presence of leptin.

The prevention of breast cancer is clearly an important goal that may be amenable to leptin-targeted therapy. In rodent models calorie restriction has consistently been reported to prevent or delay mammary tumorigenesis. However, this intervention has been implemented primarily in normal-weight animals (for review see [58]). If leptin is involved in cancer development, it is of interest to determine what happens to leptin during calorie restriction. In a long-term study of transgenic mice, MMTV-TGF-α mice (initially 10 weeks of age), two different calorie restriction interventions were used although the overall degree of restriction was similar, i.e., 25 %. In one group the restriction was implemented chronically, while in the other periods, 50 % calorie restriction (3 weeks) was followed by 100 % intake matching that of ad libitum-fed control group (3 weeks) [59]. At study termination (12 cycles of restriction/refeeding) ad libitum-fed mice had a mammary tumor incidence of 71 % compared to 35.4 % for the chronic restricted group and 9.1 % for the intermittent restricted group. Terminal serum leptin levels were highest in the ad libitum-fed mice followed by the chronic restricted mice (25 % restricted) which were reduced by 35 %. However, following 50 % calorie restriction serum leptin levels were reduced by 80 % and even after 3 weeks of refeeding remained 67 % lower compared to ad libitum-fed levels. There were no differences in leptin levels in mice with or without mammary tumors, and additionally there was no effect of either calorie restriction interventions on serum adiponectin. But after 3 weeks of 50 % restriction, the leptin/adiponectin ratio was significantly decreased. Analyses of protein expression of leptin and Ob-R in mammary tissue and mammary tumors suggested that leptin expression was reduced in mammary tissue as was Ob-R due to calorie restriction. In a complementary cross-sectional study [60], MMTV-TGF-α mice were euthanized at specific ages to assess mammary tumor status and serum leptin and adiponectin concentrations. Once again there was little effect of calorie

restriction on serum adiponectin, while intermittent restriction ameliorated age-related increases in leptin. Intermittent restricted mice consistently had lower leptin/adiponectin ratios.

Sundaram et al. [61] used C3(1)-TAG/FVBN mice, a model of basal-like breast cancer, to assess the effect of weight loss following consumption of a high-fat diet. Three groups of mice were used: (1) fed a high-fat diet (60 % fat) from 3 to 27 weeks of age, (2) fed a 10 % fat diet, and (3) fed a high-fat diet for 7 weeks and then switched to the low-fat diet. Body weight increased in high fat-fed mice, and the switch to the low-fat diet resulted in weight loss to the body weight level of the mice always fed with the low-fat diet and was then maintained at their level. Tumor latency was not affected by high-fat diet consumption or diet switch, but tumor volume was greater in the high fat-fed mice. Glucose, insulin, HOMA, and leptin levels were all higher in high fat-fed/obese mice, and all were reduced significantly in the weight loss mice to levels similar to the low-fat diet mice. In contrast adiponectin was slightly reduced in obese mice resulting in an increased leptin/adiponectin ratio in the high fat-fed mice. Although results were attributed to weight loss, the impact of low- vs. high-fat diet consumption per se should be taken into account when interpreting these results.

Leptin/Adiponectin Ratio

As mentioned above there has been interest in the leptin/adiponectin ratio with respect to the impact the interrelationship of these adipokines may have on tumorigenesis. This stems from Chen et al. [62] who reported that serum adiponectin was lower and leptin levels increased in Taiwanese women with breast cancer ($n = 100$) compared to age and body weight matched in women ($n = 100$) without cancer. They then calculated the leptin/adiponectin ratio which was significantly higher in breast cancer subjects compared to controls, and further there was a significant correlation of the ratio to breast tumor size. The increase in this ratio suggested that if there was an interaction of these two proteins particularly a protective effect of adiponectin, it would be compromised by increasing leptin levels [63]. To address this issue experimentally, Nkhata et al. [64] examined the effects of varying this ratio on proliferation rates in five breast cancer cell lines. Results were different for the various cell lines.

Another in vitro study using human breast cancer cells also evaluated interaction/relationship of adiponectin and leptin [65]. MCF-7 and MDA-MB-231 cells exposed to leptin alone (100 ng/ml) had increased-colony formation while adiponectin (10 μg/ml) alone reduced formation compared to untreated cells. Combining the two adipokines resulted in formation similar to that of controls. Similar findings were obtained using migration assays. These researchers focused on adiponectin-modulating PTP1B as the site for adiponectin's action. An additional component of this work included subcutaneous implantation of MDA-MB-231 breast cancer cells into nude mice treated with intratumoral injections of recombinant adenovirus containing adiponectin, luciferase, or saline or intraperitoneal leptin (5 mg/kg), or

leptin + the adenovirus every 36 h for 3 weeks. Their results showed that adiponectin treatment inhibited leptin-induced breast tumor growth in nude mice. Tumor growth was greatest for leptin followed by > vehicle (saline) > control adenovirus > adiponectin adenovirus + leptin > adiponectin adenovirus.

Several recent papers have addressed the leptin/adiponectin ratio in women with breast cancer. In Mexican women it was proposed that combining BMI, leptin, leptin/adiponectin, and CA 15-3 could be a biomarker for breast cancer based on a small study comparing women with ($n = 40$) or without breast cancer ($n = 48$) [66]. A cutoff point of 75th percentile was used suggesting that this tetrad identifies women at risk for breast cancer. In the microdialysis study cited earlier, Morad et al. reported that the following 6 weeks of tamoxifen treatment tumor leptin levels were reduced and adiponectin increased resulting in a decrease in the leptin/adiponectin ratio [37]. Also the leptin/adiponectin ratio was increased in Saudi Arabian women with breast cancer [27].

Leptin Antagonists

If leptin signaling is important in the development and/or progression of breast cancer, then interfering with this process would be important preventive and therapeutic approaches. Toward that goal investigators have been developing specific molecules by either modifying leptin or interfering with the leptin receptor. For a detailed recent review on this subject, see Leggio et al. [67]. Here we will highlight aspects of this area of investigation.

The laboratory of Dr. Ruben Gonzalez-Perez has developed PEG-LPrA2 an Ob-R antagonist which has been evaluated in mammary tumor models. In syngeneic model using mouse 4T1 cells implanted into Balb/c mice, local daily treatment of PEG-LPrA2 reduced tumor volume by 90 % [68]. Effects of the treatment on VEGF in tumor-bearing mice were detected. In another study MCF-7 cells were implanted in ovariectomized SCID mice treated with estradiol and MDA-MB-231 cells were implanted into intact SCID mice. When tumor size reached 100 mm^3, half the mice in each group were treated with either PEG-LPrA2 or inert control 50 μl/0.5 mM every 48 h by intravenous injection for 18 days. PEG-LPrA2 treatment reduced tumor growth from MCF-7 cells 40-fold and MDA-MB-231 twofold and reduced expression of pro-angiogenic and pro-proliferative proteins [69].

Several recent studies have used this antagonist in mice fed high-fat diets to induce obesity. Diet-induced (33 % fat diet) obese and lean (10 % fat diet) C57BL6 female mice bearing E0771 mammary tumors were tail vein if injected with PEG-LPrA2(50 μL/100 μm) once a week over 4 weeks [70]. Mice fed with this diet, as with humans, have a varied response in that some (~30 %) become obese, others overweight (30 %), and others (30 %) are resistant to obesity. The E0771 cells were injected after the mice were divided by body weight status. Results from these experiments confirm that obesity significantly contributed to tumor size, with the obese mice bearing the largest tumors and the lean ones bearing the smallest ones.

Interestingly, tumor sizes were comparable between overweight and obese mice, suggesting that being overweight already involves a level of tumor aggressiveness/progression. Mice treated with PEG-LPrA2 exhibit nonsignificant reduced tumor sizes in obese and overweight mice. Interestingly no significant effects were found in the number of tumor-associated macrophages (TAMs) in response to treatment. A significant increase in serum leptin levels was observed in obese but not in overweight mice when compared with obese-resistant mice. Corroborating in vitro experiments mimicking the E0771 tumor microenvironment of obese mice that were also presented [70] indicated that leptin was not produced by the tumor cells or macrophages but by obese adipocytes. Overall in vivo results demonstrated that in this diet-induced model leptin inhibition at the treatment dose used was insufficient to significantly reduce tumor growth. Interestingly, recent in vivo studies using the same mammary tumor model but with a 60 % high-fat diet and a more aggressive treatment scheme two injections per week instead of one resulted in significant tumor regression, suggesting a more relevant role for leptin in the same tumor model [71]. Taken together, these results illustrate how different diets can regulate differently the biological characteristics and therapeutic responses of a tumor.

Dr. Eva Surmacz's laboratory has also been working on developing leptin agonists and leptin receptor antagonists (see references 27–33 in [72]). Briefly recent work has focused on optimizing antagonists with antiproliferative effects. Allo-aca and D-Ser which does not cross the blood-brain barrier and impact body weight as did some of the earlier peptides have been studied most recently. In an in vitro study, MCF-7 cells had enhanced proliferation with the addition of leptin, but when D-Ser was included alone or with leptin, proliferation was the same as growing the cells in serum-free media.

Yuan et al. [73] used a xenograft model with MCF-7 cells and injected either PBS, leptin, or an unidentified leptin antagonist at the tumor site. Tumor size increased with leptin treatment and decreased with the antagonist compared to the PBS group. In another recent study, a leptin antagonist based on leptin-binding site 1 (LDF1)–4 amino acids was shown in vitro to reduce MCF-7 and SK-BR-3 breast cancer cell proliferation and migration. Downstream proteins were also affected, i.e., JAK2/STAT3/AKT/MAPK [74]. These investigators also studied a pegylated version of the antagonist in a xenograft model using SK-BR-3 cells. A low-dose (1 mg/kg/day) maintained tumor size and a higher-dose (10 mg/kg/day) reduced tumor growth while in control mice tumors continued to increase in size.

Leptin Advances Malignancy in Tumor Cells via Additional Intrinsic Mechanisms

In addition to inducing tumor cell proliferation, migration, and invasion, leptin has been shown to have a role in promoting a more aggressive behavior of tumor cells. For example, Chang et al. [75] elegantly demonstrated that leptin promoted a cancer stem cell (CSC) phenotype in normal and malignant human epithelial breast cells via phosphorylation of STAT3. Leptin-induced activation of STAT3 was found to

interact with the histone methyltransferase G9a; pSTAT3-G9a co-occupies promoters of noncoding RNAs such as miR-200c, resulting in their downregulated transcription. These authors also found that Ob-R is another gene derepressed by leptin-induced miR-200c downregulation, demonstrating that leptin enhances Ob-R expression through downregulation of miR-200c and that Ob-R is functionally linked to CSC traits, being highly expressed in CD24-CD44+ cells and promoting tumor sphere formation. In addition, in a diet-induced obesity rat model of breast cancer, these authors found that STAT3 blockade suppressed the CSC-like Ob-R^{hi} population and abrogated tumor progression compellingly demonstrating how targeting STAT3-G9a signaling regulates CSC plasticity in obesity-related breast cancer. Previously, Chang et al. had shown that decreased expression of miR-200c resulted in derepressing miR-200 gene targets, including ZEB1 and BMI1, to promote EMT and stem cell properties [76].

In experiments mentioned in earlier sections, Zheng et al. [54] using a different approach concluded that Ob-R-expressing tumor cells exhibited stem cell characteristics. Tumors that developed spontaneously in the MMTV-Wnt-1 transgenic mice were transplanted into obese Ob-R-deficient *Lepr^{db}Lepr^{db}* mice as well as obese leptin-deficient *Leb^{ob}Lep^{ob}* mice. The tumors that developed in the *Lepr^{db}Lepr^{db}* mice grew to eight times the volume than those in lean wild-type mice. However, tumor outgrowth and overall tumor burden were reduced in the leptin-deficient *Lep^{ob}Lep^{ob}* mice. Further residual tumors in *Lep^{ob}Lep^{ob}* mice contained fewer undifferentiated tumor cells compared with tumors in either wild-type or *Lepr^{db}Lepr^{db}* mice. In vivo limiting dilution analysis of these residual tumors from *Lep^{ob}Lep^{ob}* mice indicated reduced tumor initiating activity suggesting fewer CSCs. The tumor cell populations reduced by leptin deficiency were found to express Ob-R. These Ob-R-expressing tumor cells exhibited stem cell characteristics based on the ability to form tumor spheres in vitro, and leptin promoted their survival. These studies also implicate Ob-R as a CSC target.

In a more recent paper, Zheng et al. [55] confirmed that in both murine and human mammary cancer cells, Ob-R is necessary for maintenance and self-renewal of CSC and showed that silencing Ob-R in triple-negative MDA-MB-231 breast cancer cells resulted in the inhibition of NANOG, a master regulator of self-renewal in normal stem cells, viability of CSC, proliferation, and tumor initiation activity. These authors demonstrated that cells with silenced Ob-R reverse their EMT phenotype and exhibited a mesenchymal-to-epithelial (MET) transition, increased E-cadherin and decreased N-cadherin, and vimentin expression compared to control cells. Other authors have shown that leptin and its receptor initiate EMT via PI3K/Akt signaling and b-catenin stabilization and nuclear translocation in both ER+ and ER− breast cancer cells [43].

Leptin has also been reported to be angiogenic inducing the expression of VEGF in breast cancer cells [68, 77]. Leptin phosphorylates VEGFR-2 (the main VEGF receptor involved in tumor angiogenesis), in the absence of VEGF, upregulates Notch in endothelial and cancer cells and acts on other stromal cells such as endothelial cells and macrophages which can secrete pro-angiogenic factors [78]. It has been demonstrated that a Notch, IL-1, and leptin cross talk outcome (NILCO) is essential

for leptin regulation of VEGF/VEGFR-2 in breast cancer [78]. Molecular mechanisms of leptin pro-angiogenic actions in breast cancer could involve two waves, a short-term wave that directly transactivates VEGFR-2 in endothelial cells and a long-term wave inducing the upregulation of MMPs/TIMPs, integrins and NILCO in breast cancer cells, which positively regulate VEGF/VEGFR2 expression [79]. Thus, leptin's pro-angiogenic actions in breast cancer involve intrinsic (tumor cells) as well as extrinsic (stromal, i.e., endothelial cells, macrophages) mechanisms [80].

Another pro-tumorigenic function of leptin is its antiapoptotic activity through activation of NF-kB by leptin which promotes inflammation and survival of tumor cells [77]. STAT3 activation by leptin can further contribute to tumor cell survival [80]. Of significance, leptin uses additional mechanisms to guarantee survival of tumor cells by inducing survivin upregulation through leptin-induced activation of the EGFR-Notch 1 axis [81]. Furthermore, leptin-induced migration of breast cancer cells requires survivin which when overexpressed further increases, whereas silencing survivin abrogated leptin-induced migration of breast cancer cells [81]. Of additional interest, a recent paper by Nepal et al. [82] reported that in human breast cancer and hepatoma cells, leptin induced autophagy. Interestingly, the inhibition of autophagy blocked leptin-induced increased cell number and suppression of apoptosis, indicating a crucial role of autophagy in leptin-induced tumor progression. Moreover, gene silencing of p53 or FoxO3A prevented leptin-induced LC3 II protein expression, suggesting an involvement of p53/FoxO3A axis in leptin-induced autophagy activation. Leptin administration also accelerated tumor growth in BALB/c nude mice, which was found to be autophagy dependent. Taken together, these results demonstrate that leptin-induced tumor growth is mediated by autophagy induction [82].

Leptin Effects on the Tumor Microenvironment also Contributes to Tumor Progression

Leptin not only exerts its pro-tumorigenic action directly on tumor cells, but it can act in both autocrine and paracrine fashions on non-tumor stromal cells in the tumor microenvironment, promoting tumor progression. Adipocytes and macrophages are stromal components of the breast tumor microenvironment, and particularly in obesity, local adipose tissue plays important roles in the initiation and promotion of malignancy [83–86]. Not only is leptin produced by adipocytes and also by tumor cells, in postmenopausal women estrogen is synthesized by adipocytes, via conversion from androgens by aromatase. Given that (a) ER and Ob-R are co-expressed in breast cancer cells, (b) estrogen and leptin modulate each other [87], (c) leptin transactivates ER in the absence of estradiol through the MAPK pathway [88], (d) leptin increases the expression of aromatase in ER+ breast cancer cells [89], and since (e) estradiol increases leptin and Ob-R expression in adipose and breast cancer tissues [90], the local interactive functions of leptin and estrogen in the breast tumor microenvironment mutually reinforce and promote malignancy particularly in ER+ tumors.

Another important component of the tumor microenvironment is the presence of tumor-associated macrophages (TAMs), which have been associated with tumor progression and poor prognosis [91, 92]. Inflammatory (M1) TAMs may contribute to tumor initiation through mutation induction due to generated free radicals [93], whereas anti-inflammatory (M2) TAMs have been associated with invasion, angiogenesis, ECM remodeling, immunosuppression, and metastasis [91, 92].

In a recent comprehensive study on the effects of leptin on macrophages, a reductionist, in vitro co-culture approach was used to mimic the mammary tumor microenvironment in obese mice to analyze how adipocyte/tumor cell-derived factors in the breast may affect macrophage recruitment/functions and promote breast cancer progression [70]. The role of leptin signaling in enhancing the tumorigenic potential of tumor cells is well known; however whether leptin's signaling in macrophages induces their recruitment to the breast tumor microenvironment or whether it activates in other ways macrophage's pro-tumor functions has not been thoroughly examined. In paracrine factors such as leptin secreted by the co-culture of murine adipocytes (in vitro differentiated or ex vivo isolated from obese mouse adipose tissue), syngeneic EO771 mammary tumor cells and murine peritoneal macrophages were examined for monocyte chemotaxis and additional tumor-promoting functions. Co-culturing in vitro differentiated 3T3-L1 adipocytes with macrophages substantially enhanced leptin production by the adipocytes, even though peritoneal macrophages alone did not produce leptin. E0771 cells contrary to other mammary/breast tumor cells did not produce leptin, and co-culturing 3T3-L1 adipocytes with E0771 cells did not significantly contribute to an increase in leptin production. Interestingly, co-culturing the three cell types resulted in a profound downregulation of leptin concentration in the supernatant. In contrast, ex vivo isolated adipocytes from obese mice were the major source of leptin production. Further, as opposed to in vitro differentiated 3T3-L1 cells, obese adipocytes were not additionally stimulated to produce leptin by co-culture with either macrophages or tumor cells. On the contrary, isolated obese adipocytes cultured with either macrophages or E0771 cells significantly downregulated leptin production, and the co-culture of the three cell types resulted in a significant decrease in leptin production, as had occurred with 3T3-L1 adipocytes. Interestingly, the fact that co-culture with either macrophages or tumor cells decreased leptin production by adipocytes may suggest that in the obese mammary tumor microenvironment, leptin may be lowered. Importantly, the level of leptin production by ex vivo isolated adipocytes from obese mice was over 80-fold higher than the amount produced by 3T3-L1 in vitro differentiated adipocytes. These results suggest that leptin is more a marker of obesity than of adipocyte differentiation, since adipocytes from obese adipose tissue but not differentiated 3T3-L1 adipocytes were the major producers of leptin.

Moreover, to examine its chemotactic properties, leptin was compared with the chemokine CCL2, which is the gold standard chemoattractant for monocytes. Compared with CCL2, leptin showed a weak chemotactic activity on monocytes in vitro. These authors detected a nonsignificant chemotaxis on THP1 monocytes at the lowest leptin concentration (3 ng/ml), in agreement with others [94] who reported that leptin is a monocyte chemoattractant at concentrations as low as 1 pg/ml with

maximal effects at 1 ng/ml. But when concentrations of 10–100 ng/ml are reached, leptin chemotaxis declines. Interestingly, the fact that leptin concentrations may decrease in the breast cancer microenvironment, as shown in these in vitro co-culture studies [70], may actually result in an increase of its chemotactic activity toward monocytes/macrophages in the breast cancer microenvironment. These studies also analyzed whether leptin could also regulate macrophage M1/M2 profiles in the mammary tumor microenvironment, particularly in obesity. In contrast to the notion of leptin acting as a pro-inflammatory adipokine, these results revealed that leptin at lean and obese concentrations (20 and 100 ng/ml, respectively) decreased the expression of critical pro-inflammatory cytokines, chemokines, and reactive nitrogen species in murine peritoneal macrophages (IL-12, TNFα, CXCL2, and NO) yet significantly upregulated the pro-inflammatory and tumor-promoting IL-6, although it does not seem to modulate major anti-inflammatory molecules such as IL-10. Further leptin treatment of macrophages at 10 ng/ml significantly decreased the percentage of cells expressing F4/80 and CD11b, but it did not significantly impact Gr-1 or CD115 expression, concluding that leptin impairs the expression of myeloid differentiation markers in macrophages. Confirming its anti-inflammatory activity in these macrophages, leptin downregulated constitutive NF-kB p50 expression and constitutive and LPS-induced expression of NF-kBp65 but upregulated LPS-induced p-STAT3 expression. At low concentrations, leptin increased Notch 3 expression yet at higher concentrations it downregulated Notch 3. Interestingly, leptin did not modulate expression of STAT-1/pSTAT1 but upregulated IRAK-1 in macrophages, as previously reported [95]. Surprisingly, and in contrast to what happens in tumor cells, increasing concentrations of leptin profoundly downregulated both long (Ob-Rb) and short (Ob-Ra) forms of the leptin receptor in two different populations of macrophages: peritoneal and tumor-associated macrophages (TAMs) [70].

Tumors recruit stromal fibroblasts from the microenvironment in a process referred to as the desmoplastic reaction, and these carcinoma-associated fibroblasts (CAF) are reprogrammed to produce growth factors, cytokines, and ECM-remodeling proteins that act in autocrine and paracrine fashion to support tumor proliferation, migration, and invasion. It was recently shown that Ob-R RNA expression and leptin secretion occur in CAFs, indicating a novel integral role for leptin in mediating the bidirectional cross talk between ER+ breast cancer cells and CAF driving tumor growth via leptin-mediated activation of ER in tumor cells resulting in proliferation and invasion in breast cancer cells [47].

Leptin's Role in Inflammation, Chemotaxis, and Adaptive Immunity Impacts the Cancer Host's Immune Responses

Leptin plays important roles in both adaptive and innate immunities as evidenced by Ob-R expression in immune cells [96], and leptin-deficient humans exhibit impaired immunity [97]. Earlier reviews have extensively addressed the role of

leptin and immunity [98–102]. With regard to innate immunity, leptin is a direct potent chemoattractant for monocytes and macrophages, although the presence of full-length receptors on migrating cells is required [94]. In addition, leptin increases the recruitment of blood monocytes via adipose-tissue-derived endothelial cells by stimulating the upregulation of EC adhesion molecules necessary for the diapedesis of the monocytes [98]. Acting on monocytes leptin induces the release of other cytokines such as tumor necrosis factor alpha (TNF-α) or interleukin-6 (IL-6) as well as CCL2 and VEGF [99]. Leptin is also able to stimulate the chemokinesis of eosinophils [100] and the chemotaxis of neutrophils [101]. Most of these effects are mediated through the long signaling form of the leptin receptor, Ob-Rb, which is expressed mainly by endothelial cells and leukocytes. In adaptive immunity, leptin enhances T-cell proliferation and Th-1 pro-inflammatory cytokine production in vitro, whereas little is known about the effect of this adipocytokine on the migratory behavior of T cells.

Leptin has far reaching effects on the immune system. For example, leptin activates dendritic cells licensing them for Th-1 priming and increasing migratory performance [102]. Also obese mice display decreased thymic function and increased inflammatory responses. With expression of Ob-R on T cells and supporting thymic epithelium, aberrant signaling through Ob-R has been thought to be the direct cause of thymic involution in obese mice [103]. Recently Sreenivasan et al. [104] demonstrated that the absence of Ob-R on either thymic epithelial or T cells did not lead to the loss of thymic function, indicating that the thymoprotective effect of leptin was mediated by obesity suppression rather than direct signaling to the cellular components of the thymus. Using the Cre-Lox system to specifically excise Ob-R from both the epithelial and lymphocytic compartments of the thymus, these researchers showed that while global Ob-R deficiency resulted in thymic involution, thymic specific loss of Ob-R did not alter thymus function. These results support that the thymic involution in leptin-deficient mice reflects indirect effects of obesity rather than the loss of a direct thymoprotective function of leptin [104]. On the other hand, and in contrast to these results, analyzing mammary tumor-bearing mice which exhibited a profound thymic involution, Lamas et al. [105] very recently found that these mice had profound thymic involution possibly associated with the accumulation of adipocytes [105]. A significant number of adipocytes around and infiltrating the tumor bearers' thymuses were observed. While there were no changes for adiponectin in the thymuses of either normal or tumor-bearing mice, significantly higher levels of leptin were detected in the thymocytes of tumor bearers. This was correlated with an increased expression of some cytokines (IL-2, IFN-γ, and GM-CSF). Coculture of thymocytes from normal mice with ex vivo obtained adipocytes from tumor bearers gave similar results. These findings suggest that infiltration and accumulation of adipocytes into the thymuses of tumor-bearing mice may play an important role in their altered morphology and functions [105].

Leptin and Non-breast Malignancies

Accumulating evidence supports that leptin is also a link between obesity and the increased incidence of various non-breast cancers, although leptin and its receptor Ob-R are also expressed in numerous tumors regardless the obesity status of the host/patient. Ob-R is highly expressed on multiple malignant cells, including those of liver, colorectal, pancreatic, esophageal, gastric, prostate, and ovarian origin, as compared with their normal counterparts (in which the receptor is expressed at very low levels or not at all). Thus the elevated serum leptin levels associated with obesity could play a role in these various cancers. For example, Stefanou et al. [106] reported that leptin's expression was highly correlated with human telomerase reverse transcriptase (hTERT) expression levels in hepatocellular carcinoma (HCC). Further, in HCC (HepG2) cells they reported that leptin-induced upregulation of hTERT and telomerase activity was mediated through binding of STAT3 and Myc/Max/Mad network proteins on the hTERT promoter. Their work also revealed that leptin could affect HCC progression and invasion through its interaction with cytokines and MMPs in the tumor microenvironment and that histone modification contributed to leptin's gene regulation in HCC. These authors concluded that leptin is a key regulator of the malignant properties of HCC cells through modulation of hTERT, a critical player of oncogenesis. Moreover, Saxena et al. have confirmed that the role of leptin in cancer cell proliferation, invasion, and metastasis is attributable to the activation of several signal transduction pathways in HCC [107].

Obesity and obese-related chronic low-grade inflammation have been reported to promote colorectal cancer (CRC) development [108]. In CRC, leptin was shown to be a potent mitogen and antiapoptotic cytokine and promoted the invasiveness of familial adenomatous colonic cells. It has been shown that leptin expression dramatically increases from normal colonic mucosa to adenoma and adenocarcinoma, suggesting its involvement in multistep colorectal carcinogenesis [109]. Hiu et al. [110] documented that leptin was overexpressed and bound to its receptor in CRC tissues. In a case-control study conducted in the USA, men with colorectal adenomas had elevated circulating leptin levels [111]. Another recent study of 3614 CRC vs. 1215 colorectal adenomas vs. 5220 controls found a positive association between serum leptin and the colorectal adenoma risk, but not with CRC risk [112]. Other researchers also reported that serum leptin levels were not significantly correlated with increased CRC burden when factors such as BMI and waist circumference were taken into consideration [113]. The relationship between serum leptin concentrations and CRC progression and aggressiveness has also been reported by Tutino et al. [114], who found that the expression of leptin, Ob-Rb, and VEGF correlated positively with the grade of tumor differentiation. These findings led the authors to suggest that binding of leptin to Ob-Rb stimulated the proliferation of CRC cells resulting in tumor overgrowth. The synergistic function of leptin and VEGF may accelerate angiogenesis and promote cancer invasion and metastasis of carcinoma tissues to other sites and tissues in CRC patients.

The potential link between elevated serum leptin concentrations and CRC progression and invasiveness may depend on different interrelated factors such as gender, metabolic syndrome, and/or genetic background. For example, Stattin et al. [115] reported that an increased level of serum leptin was related to the invasiveness of CRC in men, but not in women. Another study associated the aggressiveness of CRC tumors in males with high-serum leptin levels and hypertension as well as raised plasma glucose, triglycerides, and HDL cholesterol concentrations [116]. On the other hand, several investigators have found higher leptin levels associated with the development of CRC in women. For example, Ho et al. [117] found a relationship between serum leptin levels and progression of CRC in postmenopausal women although the effect of hyperleptinemia was mediated mostly by insulin. Tamakoshi et al. [118] also reported that the invasiveness of CRC in women was associated with a high-serum leptin level. Other authors reported that only very high levels of leptin and other metabolic factors acting simultaneously (levels of C-peptide, HbA1c, and leptin/Ob-R ratio) activated and increased CRC proliferation [119]. Thus, it can be concluded that the relationship between obesity, leptin's serum levels, and CRC is multifactorial.

Experimental studies in mice support a role for leptin in the development of CRC. For example, leptin-deficient $Lep^{ob}Lep^{ob}$ mice were significantly less sensitive to azoxymethane-induced polyp formation than wild-type mice [120] suggesting a role for leptin in stimulating initiation of colon cancer. Furthermore, a comparison of wild-type and $Lep^{ob}Lep^{ob}$ mice fed a high-fat diet showed that $Lep^{ob}Lep^{ob}$ mice were protected from polyp formation compared to wild-type mice [120]. In vitro experiments using the human CRC cell line HCT-116 showed that leptin activated the PI3K-AKT pathway resulting in increased cancer cell proliferation [121]. This growth-stimulatory effect of leptin was mitigated by treatment with the PI3K inhibitor LY294002. Notably, others have argued that the Jak2-STAT3-activating capacity of leptin is responsible for stimulating CRC cell proliferation [122]. However, these pathways are clearly not mutually exclusive, and these findings could simply imply that therapies targeting multiple pathways might be needed in certain patient populations. According to these authors, the leptin-signaling pathway in CRC is mainly transduced by JAK/STAT, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3 kinase (PI3K), mTOR, and 5′AMPK signaling pathways.

Leptin signaling has also been implicated to have a role in gastric cancer. Recently, the expression of leptin and its receptor, Ob-R, was assessed among patients with gastric adenoma ($n=38$), early gastric cancer ($n=38$), and advanced gastric cancer ($n=38$), as a function of their clinicopathological characteristics [123]. Leptin was expressed in gastric adenomas (42.1 %), early gastric cancer (47.4 %), and advanced gastric cancer (43.4 %), and Ob-R expression tended to increase from gastric adenoma (2 %) to early gastric cancer (8 %) and to advanced gastric cancer (18 %), respectively. Gastric cancer cell lines were also investigated and leptin induced the proliferation by activating STAT3 and ERK1/2 and upregulating the expression of VEGF. Blocking Ob-R with pharmacological inhibitors and by RNAi decreased both the leptin-induced activation of STAT3 and ERK1/2 and the leptin-induced expression of VEGF. The authors concluded that leptin plays a role in gastric cancer by stimulating the proliferation of gastric cancer cells via activating the STAT3 and ERK1/2 pathways [123].

With regard to ovarian cancer, a study of epithelial ovarian cancers in Middle Eastern women indicated that up to 60 % of these tumors overexpressed Ob-R and that this upregulation correlated with reduced progression-free survival [124]. Investigations using the OVCAR-3 ovarian cancer cell line showed that leptin signaling inhibited apoptosis and stimulated cell division via inhibition of p21 and increased expression of cyclin D1 and myeloid cell leukemia sequence 1 [125, 126]. Interestingly, exposure to bisphenol A, a common molecule found in many plastics, increased Ob-R expression and inhibited caspase-3 expression and activity in ovarian cancer cell lines [127]. Moreover, the inhibition of caspase-3 activity was augmented by exposure to estrogen. Together, these observations demonstrated a direct role for leptin in the growth and survival of ovarian cancer cells, but further in vivo studies are needed to confirm the relevance of these findings to the clinical setting.

In another gynecological malignancy leptin contributed to tumor progression by inducing a decrease in apoptosis in human endometrial cancer cells, partly through nuclear factor NF-kB activation via phosphorylation in the IKK/NIK pathway [128]. Inhibition of either IKK or NIK partly neutralized this suppression of apoptosis. Expression levels of Ob-R and IKK/NIK signaling proteins were higher in poorly and moderately differentiated than in well-differentiated EC tissues, and higher Ob-R expression was observed in clinical stages II and III, compared with stage I EC ($P=0.012$). High-serum leptin concentration displayed mild correlation ($r=0.23$, $P=0.035$) with the degree of EC differentiation [128].

The circulating levels of leptin in renal cell carcinoma (RCC) patients have been the primary focus of numerous epidemiological studies highlighting the strong correlation between elevated leptin concentrations and increased risk of RCC [129]. Ob-R was expressed in renal tissue as well as in RCC cell lines supporting a role for leptin signaling in RCC carcinogenesis. Interestingly, increased circulating levels of leptin and the overexpression of Ob-R have both been associated with the invasion and progression in of human RCC [130, 131]. In the same lines, Li and colleagues recently demonstrated that leptin stimulated cell proliferation and promoted the invasion and migration capabilities of RCC Caki2 cells upon the activation of both extracellular signal-regulated kinase (ERK) and JAK/STAT3 signaling pathways [132].

Prostate cancer is the most commonly diagnosed malignancy in men and shows a predilection for metastasis to distant organs. The possible role of obesity and/or leptin and prostate cancer was initially not clear [133–135]. For example, Stattin and coworkers published one paper indicating serum leptin levels that were associated with prostate cancer and then a second that found no relationship [136, 137]. A recent in vitro study by Huang et al. [138] indicated that leptin increased the migration of human prostate cancer cells and the expression of avb3 integrin on these cells. Leptin-mediated migration and increased integrin expression were attenuated by the addition of an Ob-Rb antisense oligonucleotide. The activation of insulin receptor substrate (IRS-1), PI3K, Akt, and NF-kB pathways after leptin treatment was demonstrated. Furthermore, leptin-induced integrin expression and migration activity were inhibited by specific inhibitors (small interfering RNAs (siRNAs) and mutants of the IRS-1, PI3K, Akt, and NF-kB cascades). This study showed that leptin stimulated the migration of human prostate cancer cells, and one of the

mechanisms underlying leptin-directed migration was transcriptional upregulation of avb3 integrin expression through the Ob-Rb/IRS-1/PI3K/Akt/NF-kB signal transduction pathway. These findings are consistent with obesity and high-serum leptin levels being associated with aggressive prostate cancer.

Glioblastoma multiforme is the most malignant primary tumor of the central nervous system, with significant resistance to chemotherapy. Han et al. [139] found that glioblastoma cells that were resistant to temozolomide treatment exhibited high expression of Ob-R. Ob-R can serve as a marker to enrich glioblastoma cells with some stem/progenitor cell traits, which explained the reason for this resistance. The study also showed that STAT3-mediated SOX2/OCT4 signaling axis maintained the stem/progenitor cell properties of Ob-R+ cells, which indirectly regulated glioblastoma temozolomide resistance. These findings provide insight into the molecular link between obesity and glioblastoma. Further a better understanding of this drug-resistant population may lead to the development of more effective therapeutic interventions for glioblastoma [139].

Conflicting data have been reported for the relationship of leptin and the development of hematological malignancies. For example, a case-control study done in Greece including 95 patients with incident B-chronic lymphocytic leukemia and 95 hospital controls matched for age and gender was studied between 2001 and 2007, and blood samples were collected. Serum levels of leptin were found to be inversely associated with disease risk [140]. In contrast, in human acute myelogenous leukemia cells, leptin promoted leukemia cell growth by activating STAT3 and MAPK, although not directly dependent on ERK [141].

Conclusions

Twenty years after the first publications introducing the world to leptin, there is still intense interest in this protein. There have been recent reviews focused on leptin and metabolism [142] and leptin's potential effects on cancer in general [143, 144]. Other reviews have been specific to breast cancer [145–147]. Overall it is clear that leptin is an important protein with pleiotropic effects acting on different pathways and it is involved in many physiological processes. In cancer some of its effects may be further enhanced by elevated levels due to obesity. Alterations in gene expression of its receptors may also provide insights into its role in various cancers. Ongoing work in numerous laboratories will continue to identify and clarify leptin's actions, some of which will hopefully help in cancer prevention and/or treatments. Presently therapeutic approaches that can be used to prevent or slow the tumorigenesis process by interfering with leptin's growth promoting actions are being pursued.

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Chapter 4
Leptin-Signaling Pathways as Therapeutic Targets in Cancer

Neeraj K. Saxena and Dipali Sharma

Abstract Evidences from molecular, clinical, and epidemiological studies have shown that high plasma level of leptin correlate with obese state and is emerging as a key adipocytokine mediating the molecular effects of obesity on cancer. Dysregulation of leptin influences various stages of carcinogenesis from initiation and growth to metastatic progression. In addition to activating its canonical signaling, leptin is now known to functionally interact with multiple oncogenic pathways. This "hyperactive leptin-signaling network" in cancer cells leads to simultaneous activation of multiple oncogenic pathways leading to increased proliferation, decreased apoptosis, acquisition of mesenchymal phenotype, and enhanced migration and invasion potential of tumor cells. Leptin is also known to interact with other important molecular effectors such as estrogen, IGF-1, insulin, VEGF, and inflammatory cytokines and achieve a wider impact across various tumor types. Development of a better understanding of leptin-signaling network has provided multiple therapeutic opportunities to inhibit important nodes of this network. This article presents an overview of the studies investigating the involvement of leptin and hyperactive leptin-signaling network in cancer progression and strategies to inhibit leptin signaling in cancer.

Keywords Anti-leptin-receptor monoclonal antibodies • Leptin muteins • Honokiol • Adiponectin receptor agonist

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There is increasing epidemiological evidence that pathophysiological effects of obesity are largely mediated by adipocytokine dysregulation [1–3]. Previously regarded as just "inert energy storing cells," adipocytes are now known for their role as "active endocrine organs" secreting ~20 adipocytokines, traditionally involved in energy homeostasis. Leptin is one of the important adipocytokines that have attracted considerable attention since its identification in 1994 [4], first as a satiety hormone and subsequently for its apheliotropic actions, its role in the pathogenesis of atherosclerotic vascular disease and importantly carcinogenesis [5–7]. This review examines leptin and its signaling pathways in relation to the development and progression of cancer and discusses various strategies to modulate leptin-signaling axis for therapeutic benefits.

Connection Between Dysregulated Leptin Levels and Cancer

Synthesized and secreted predominantly from preadipocytes and adipocytes, leptin circulates as a 16 kDa non-glycosylated protein partially bound to plasma proteins and exerts its actions on hypothalamus and various peripheral organs, including the liver, skeletal muscle, and pancreas [8–10]. Leptin is the most abundant adipokine, known for its pleiotropic actions, and its levels are increased in obese state. Recent advances in leptin research, accumulating evidences from preclinical, clinical, and epidemiological studies, have shown its role and importance in growth and progression of various cancers. While majority of studies show positive associations between leptin and various cancer types, some studies have also found contradictory associations.

Elevated serum leptin levels significantly correlate with poor clinicopathological characteristics for postmenopausal breast cancer patients with ER positive breast cancer [11]. Overexpression of leptin was observed in 92% of breast tumors whereas none of the normal breast epithelium expressed leptin. Also, exhibiting the importance of leptin receptor, it was shown that leptin receptors were not detectable in normal mammary epithelial cells while 83% of breast cancer cells exhibited high expression of leptin receptor (LR) [12]. Leptin and leptin receptor were also found to be overexpressed in primary and metastatic invasive ductal breast carcinoma [13, 14]. Although high leptin levels are characteristically present in obese state and are known to mediate the molecular effects of obesity, a case-control study showed that high leptin levels associate with breast cancer risk even after adjustment of obesity indices. This study clearly showed that high leptin levels might have an independent oncogenic role in breast cancer [15]. When leptin levels were evaluated in obese patients with newly diagnosed breast cancer, without diabetes and compared with obese women without breast cancer and diabetes, higher leptin levels were observed in obese women with breast cancer as compared to obese women without breast cancer [16]. A meta-analysis of data from 23 studies involving 2058 breast cancer patients, 2078 healthy controls, and 285 breast benign controls showed that circulating levels of leptin were highest in lymph node metastasis-positive patients followed by breast cancer patients, breast benign diseases, respectively, with the lowest levels observed in healthy people [17].

The relationship of leptin levels with prostate cancer appears to be complex with some studies reporting a positive association between increased serum leptin levels and elevated prostate cancer risk while others found no change [18]. While no correlation was found between higher BMI and prostate cancer incidence, higher BMI correlated with increased prostate cancer-associated mortality [19]. Further analyses revealed that high BMI is associated with high-grade prostate cancer suggesting that pathogenesis of high-grade disease is linked with obesity-driven signaling [20]. Meta-analysis of 16 published studies comprising of 6569 cases and 8405 controls for the leptin-receptor mutation (G2548A) exhibited significant association with an increased risk of prostate cancer [21]. Interestingly, prostate cancer patients with larger disease burden had higher serum leptin concentrations than patients with lower disease burden even after stratifying for other variables such as age, testosterone level, height, and BMI [22] indicating that leptin might be an important oncogenic mediator of prostate cancer.

Elevated leptin levels were positively correlated with colorectal cancer [23–25]. Analysis of relapse-free survival and overall survival showed that higher serum leptin level was an independent predictor for adverse outcome in colorectal cancer [26]. Higher expression of leptin and leptin receptor were found to be associated with larger tumor size, nodal metastasis, advanced stage, and lower disease-free survival for thyroid cancer patients [27, 28]. Meta-analysis of six studies with 3136 individuals showed that high leptin level was associated with increased risk of endometrial cancer even after adjusting for confounding factors indicating that high leptin level is an independent risk factor for endometrial cancer [29]. Using immunohistochemical analysis, overexpression of leptin and leptin receptor was observed in endometrial cancer and was found to be associated with malignancy, invasion, and metastasis [30]. Collectively, there is strong epidemiological and molecular evidence supporting the role of leptin in cancer growth and progression.

Effect of Leptin on Cancer Progression and Metastasis

Cancer growth and metastatic progression is a complex multistep process. Leptin is known to alter the balance between proliferation and apoptosis leading to increased tumor growth as well as support epithelial-mesenchymal transition enabling cancer cells to gain increased migration and invasion potential in various cancer types.

Studies utilizing leptin-deficient (ob/ob) or leptin-receptor-deficient (db/db) mice crossed with MMTV-transforming growth factor-α (MMTV-TGF-α) show that MMTV-TGF-α/Ob/Ob and MMTV-TGF-α/db/db does not develop oncogene-induced mammary tumorigenesis (Cleary MP, genetically, 2003 and Cleary MP, Leptin receptor, 2004) clearly exhibiting the integral role of leptin and leptin receptor in spontaneous mammary tumorigenesis. High-fat diet (HFD)-induced obese MMTV-TGFα mice show elevated leptin levels as well as increased tumor growth [135]. Leptin treatment significantly increases tumor growth in athymic nude mice [31] whereas genetically obese Zucker rats possessing leptin-receptor defect does

not develop carcinoma after chemical carcinogen injection [32]. Studies utilizing various in vivo models have shown that intact leptin signaling is required for mammary tumor development. Leptin has been shown to interact with leptin receptors present on breast cancer cells and activates several intracellular signaling pathways. Leptin inhibits apoptosis and increases growth of breast cancer cells via activating JAK/Stat3, ERK (extracellular signal-regulated kinases 1/2) and Akt signaling pathways [33–35]. Leptin is known to support angiogenesis by inducing the expression of vascular endothelial growth factor (VEGF), VEGF receptors (VEGFR1 and VEGFR2) [36–39]. By transactivating EGFR and exhibiting bidirectional cross talk with IGF-1R, leptin potentiates growth as well as invasion and migration of breast cancer cells [40]. Recent discoveries show that leptin can also induce epithelial-mesenchymal transition of breast cancer cells by activating Wnt-catenin pathway in a MTA1-dependent manner [41]. These are important observations as they demonstrate that leptin can activate MTA1 to support EMT in breast cancer cells priming them for invasive behavior. Additionally, leptin also increases the expression of anti-apoptotic protein, survivin achieved by activating Notch signaling pathway. Survivin presents a critical node in leptin action as silencing of survivin inhibits leptin-induced growth activation [31]. Furthermore, leptin modulates phosphorylation of estrogen receptor by ERK activation leading to estrogen-independent growth of breast cancer cells [42, 43]. Yet another way leptin activates growth of breast cancer cells is by stimulating aromatase expression leading to increasing estrogen levels [44].

Prostate cancer cells express leptin receptors and are responsive to leptin stimulation. Several studies have shown that leptin treatment stimulates the growth of prostate cancer cells [45, 46], with androgen-insensitive cells (DU145 and PC3) showing a stronger response than androgen-sensitive cells (LNCap) which appear to be more refractory [46]. Leptin activates several pathways such as PI3K, MAPK and JNK (c-Jun N-terminal kinase) to affect growth to prostate cancer cells [46–48]. Leptin has been shown to induce the expression of VEGF, transforming growth factor β1 and basic fibroblast growth factor and stimulate proliferation and angiogenesis in prostate cancer cells [49, 50]. Differential effects of leptin on the invasive potential of prostate cancer cells have been proposed based on androgen-dependent or -independent status [51]. Interestingly, both long-form and short-form leptin receptors have been shown to be involved in promoting growth of prostate cancer cells [51]. Another study implicated the involvement of $\alpha\nu\beta3$ integrin in leptin-induced migration of prostate cancer cells [52]. In vivo studies utilizing athymic nude mice xenografted with LAPC-4 cells show that high-fat diet stimulates tumor growth in comparison to low-fat diet [53].

The association between leptin and colorectal cancer (CRC) remains somewhat unclear with some studies showing a positive association between leptin and CRC [23–25] while others showing a negative correlation [13, 54]. Leptin has been reported to stimulate growth of human colon cancer cells, but interestingly hyperleptinemia does not promote tumor progression in nude mice model or Apc(Min/+) model [55]. Human colon cancer cells and colonic tissue express

leptin receptor and stimulation with leptin increases phosphorylation of ERK and cell proliferation in vitro as well as in vivo [56]. Leptin and leptin receptor are overexpressed in gastric cancer tissues indicating that locally elevated leptin signaling may act in a paracrine or autocrine manner and stimulate proliferation of gastric cancer cells [57, 58]. Mechanistically, leptin activates Stat3 and ERK pathways in gastric cancer cells, and leptin-stimulated Stat3 activation is independent of ERK activation. Leptin stimulates SHP2 phosphorylation and increases Grb2 binding to SHP2 leading to ERK activation and SHP2 silencing abolishes leptin-induced ERK activation. Exhibiting hierarchy of signaling events, JAK/Stat3 inhibition reduces ERK activation as well as gastric cancer cell proliferation while SHP2 inhibition only partially reduced cell proliferation [57]. Also, leptin increases invasion potential of kidney and colonic epithelial cells by activating PI3K, rho-, and rac-dependent signaling pathways, and leptin-induced invasion can be blocked with inhibition of these pathways [59]. Leptin has been shown to increase proliferation and inhibit apoptosis in several cancer types. A study investigating the effect of leptin on proliferation and apoptosis in human esophageal adenocarcinoma cells found that exogenous leptin increases cell proliferation but has no effect on apoptosis and necrosis [60]. Using mature adipocytes and preadipocytes isolated from C57BL/6 and leptin-deficient ob/ob mice, it is shown that adipocytes increase proliferation of colon cancer cells in vitro, indicating that adipose tissue may directly promote growth of colorectal cancer, in part via leptin [61]. Studies investigating the effect of leptin on lung cancer cells show that leptin promotes proliferation of lung cancer cells and inhibit endoplasmic reticulum stress-related apoptosis [62] by modulating PERK and ATF6 [63]. Leptin also promotes metastasis of lung cancer cells by supporting EMT in a TGF-β-dependent manner [64].

Leptin is shown to have a strong positive association with incidence of endometrial cancer in a case-control study of endometrial cancer in Greece [65]. Leptin receptor is aberrantly expressed and elevated serum leptin levels are found in endometrial cancer indicating possible involvement of leptin-leptin receptor in pathogenesis of endometrial cancer [66]. Leptin promotes growth and invasion potential of human endometrial cancer cells and JAK/STAT and AKT pathways act as critical mediators of leptin action [67]. Analysis of endometrial cancer tissues shows the association between HIF-1α, leptin, and leptin receptor. HIF-1α associates with overexpression of leptin and leptin receptor in endometrial cancer tissue. These study show that leptin can exert autocrine effect to stimulate endometrial cancer progression [68]. Furthermore, leptin treatment reduces the number of endometrial cells in G0/G1 phase and increases cell population in S-phase. Leptin increases cyclin D1 expression and decreases cyclin-dependent kinase inhibitor p21 (WAF1/Cip1) expression in ishikawa cells via Stat3 [69]. Leptin also regulates VEGF/VEGFR2 in human endometrial cancer cells and promotes angiogenesis. Interestingly, effect of leptin on proangiogenic molecules is more pronounced in malignant cells versus benign cells [70].

Increasing leptin concentration is also associated with pancreatic cancer [71] warranting further studies to analyze the molecular mechanism underlying leptin and pancreatic cancer cell growth. A recent study shows the presence of functional leptin receptor in pancreatic cancer cells [72]. Leptin treatment increases migration and invasion of pancreatic cancer cells by upregulating the expression of matrix-metalloproteinase-13 (MMP-13) in a JAK/Stat3-dependent manner [73]. Leptin stimulates tumor growth and lymph node metastasis in subcutaneous and orthotopic in vivo models. Analyses of human pancreatic cancer tissue show that the expression of leptin receptor positively correlates with MMP-13 expression and increased leptin-receptor and MMP-13 expression correlates with lymph node metastases and higher tumor grade [73]. PI3K/AKT pathway has also been shown to be involved in leptin-induced pancreatic cancer growth and increased migration potential [72]. Inhibition of PI3K or depletion of leptin receptor inhibits growth of cancer cells in an orthotopic model [72]. In vivo orthotopic murine pancreatic cancer model also shows increased tumor burden in the diet-induced obese mice as compared to lean mice indicating the involvement of leptin [72]. HIF-1α directly activates the expression of leptin receptor in pancreatic cancer cells and xenograft models. The correlation between HIF-1α and leptin receptor with clinicopathological characteristics of pancreatic cancer samples further shows the importance of HIF-1α –Ob-Rb axis in pancreatic cancer [74].

Leptin as a Regulator or an Important Participant of Various Oncogenic Pathways

Canonical Signaling Pathways of Leptin

Leptin binds to the extracellular domain of specific membrane receptor (leptin receptor) to exert its cellular functions [75]. Localized to the cell membranes, present on a variety of tissues, leptin receptors are class I cytokine receptors typically containing a cytokine receptor homologous domain in the extracellular region [75]. N-terminus of leptin receptor contains two conserved disulfide links and WSXWS motif is present in the C-terminus. Leptin receptor has six isoforms that have similar extracellular ligand-binding domain at the amino terminus but different intracellular carboxy-terminal domains. Only one leptin-receptor isoform, known as the “long form,” has the intracellular motifs necessary for activation of intracellular signaling events, while five other isoforms, collectively known as the “short forms,” exhibit transmembrane domain and truncated intracellular domains (Fig. 4.1) [75–77]. Similar to other class I cytokine receptors, intracellular signals of leptin are transmitted mainly by the JAK-Stat3 signaling. JAKs associate constitutively with the conserved box 1 and 2 motifs in the intracellular domain of long-form leptin receptor. Binding of leptin to leptin receptors leads to JAK2 activation which in turn

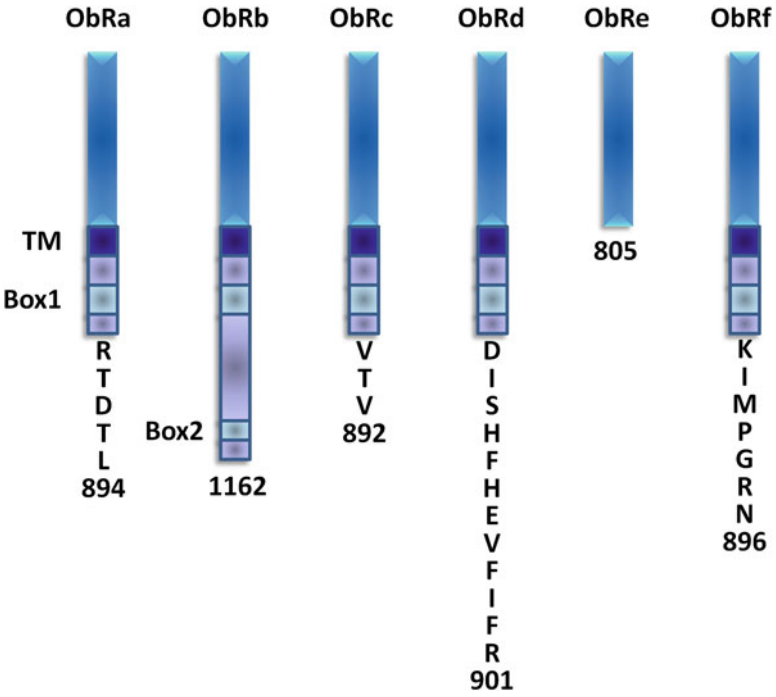

Fig. 4.1 Structure of alternatively spliced leptin receptors. Leptin receptor has six isoforms denoted here as ObRa, ObRb, ObRc, ObRd, ObRe, and ObRf. All leptin receptors share a common extracellular leptin-binding domain, but differ at the carboxy-terminus intracellular domain. Terminal amino acid residues for various LEPR isoforms are denoted by the *alphabet code*. Only the long isoform ObRb has complete intracellular motifs necessary for JAK-Stat signaling. In addition to intracellular domain, ObRe also lacks a transmembrane domain (TM) and hence circulates as a soluble receptor

phosphorylates Tyr-985, Tyr-1077 and Tyr-1138 in the cytoplasmic domain of leptin receptor [78]. JAK2/Stat3 activation by leptin is an important node in leptin-signaling cascade [79, 80]. Once activated, phosphorylated Stat3 dimerizes and translocates to nucleus followed by binding to putative Stat3-binding motifs (GAS sites) in the promoter region of Stat3-responsive genes. ERK is another major kinase that is known to get upregulated in response to leptin, mediated by either SHP2 from Tyr-985 of leptin receptor or directly from JAK2 [81]. Physiologically, leptin signal is terminated by induction of SOCS-3 and protein-tyrosine phosphatase 1B (PTP1B). SOCS3 belongs to a family of proteins that inhibits the JAK-Stat pathway. Structurally, SOCS proteins consist of a variable N-terminal domain, a central SH2 domain, and a C-terminal domain called SOCS-box motif [82]. SOCS gets activated by cytokines and inhibit the receptor acting in a negative feedback loop. It has been shown that SOCS3 overexpression inhibits leptin signaling. PTP1B recognizes a specific substrate motif of JAK2 and dephosphorylates JAK2 resulting in leptin-signaling inhibition [83].

Noncanonical Signaling-Functional Interactions of Leptin with Multiple Pathways

Using various in vitro and in vivo models, we and others have shown that leptin regulates multiple molecules and oncogenic pathways involved in proliferation, adhesion, invasion, migration, inflammation, and angiogenesis (Fig. 4.2), such as cyclin D1, survivin, β3 integrin, interleukin-1 (IL-1), IL-1 receptor, vascular endothelial growth factor, and its receptor type 2 [31, 33, 40, 84–89]. Simultaneous activation of multiple signaling pathways including ERK, Akt, and Stat3 network via leptin broadens its biological impact (Fig. 4.3) [33, 40, 67, 84, 90]. Regulation of transactivation function of Stat3 enables leptin to regulate the expression of various important genes. Leptin directly influences the transactivation function of many coactivator molecules to directly alter the local chromatin structure. Leptin is known to increase histone acetylation at the cyclin D1 promoter. Genes in repressed state are associated with methylation of K9-dimethylated H3 while the active state shows increased methylation of K4-dimethylated H3 [91]. Leptin influences histone methylation at the promoter region, increasing H3-K4 methylation and decreasing H3-K9 methylation to allow recruitment of specific coactivator complexes

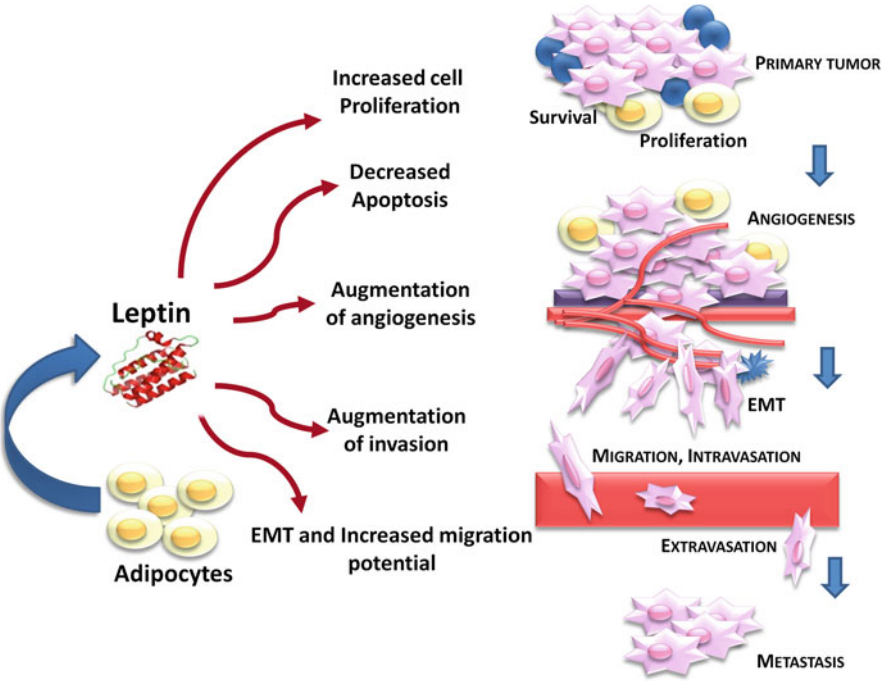

Fig. 4.2 Leptin affects tumor growth and progression. Leptin increases proliferation, decreases apoptosis, augments angiogenesis, and increases invasion and migration. Leptin supports primary tumor growth and metastatic progression

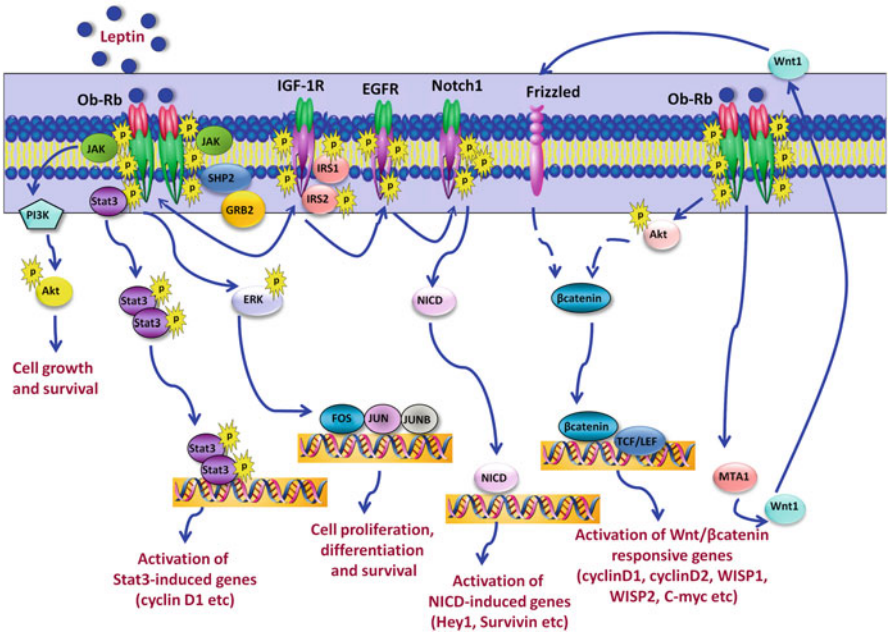

Fig. 4.3 Complex leptin-signaling network. Binding of leptin with the long form of leptin receptor (Ob-Rb) results in conformational changes and receptor oligomerization. Leptin receptor gets phosphorylated at multiple sites followed by JAK activation which in turn phosphorylates Ob-Rb at additional sites. These early events in leptin signaling trigger activation of multiple signaling pathways such as Akt activation, ERK phosphorylation, and Stat3 activation. Bidirectional cross talk occurs between Ob-Rb and IGF-1R where leptin phosphorylates Ob-Rb as well as IGF1-R. Leptin also transactivates EGFR and activates Notch1 resulting in release of NICD which induces Notch-responsive genes. Leptin increases the expression of MTA1 resulting in increased levels of Wnt1 and activation of Wnt1/β-catenin network. *AKT* protein kinase B, *GRB2* growth factor receptor-bound protein 2, *JAK* Janus kinase, *Ob-R* leptin receptor, *MAPK* mitogen-activated protein kinase, *PI3K* phosphatidylinositol 3 kinase, *SHP2* Src homology 2-containing tyrosine phosphatase, *STAT3* signal transducer and activator of transcription 3; insulin-like growth factor 1 receptor, *IGF-1R* epidermal growth factor receptor, *EGFR* transcriptionally active intracellular Notch, *NICD* metastasis associated protein 1, MTA1

including Med1 and SRC1 resulting in increased gene expression [33]. Leptin controls various genes that have important regulatory roles in growth and apoptosis such as cyclin D1 and survivin in cancer cells [92–94] and luminal epithelial cells of mouse MMTV-Wnt1 mammary tumors [95]. The expression of survivin, a member of inhibitor-of-apoptosis proteins (IAP) family, is also increased by leptin in cancer cells [31, 96, 97]. We show that leptin-mediated increased survivin expression involves a complex upstream signaling network consists of activation of EGFR-Notch1 axis. These studies show leptin-mediated activation of Notch1 and induction of the recruitment of NICD (transcriptionally active intracellular Notch) to survivin promoter. It is interesting to note that leptin-induced Notch 1 activation is mediated in part by epidermal growth factor receptor (EGFR) transactivation

implicating the involvement of another oncogenic pathway in leptin network [31]. EGFR transactivation in leptin function has also been reported in gastric cancer cells [98] and esophageal adenocarcinoma cells [99]. Many other studies have shown various signaling cross talks between leptin and IGF1, IL6, Notch, and sex hormones [31, 40, 100, 101] leading to increased growth and metastatic progression of cancer cells. Together, it is important to acknowledge that the highly active leptin-induced signaling network (Fig. 4.2) is involved in many different aspects of tumorigenesis and metastasis partly achieved by its functional interactions with multiple molecular mediators and pathways.

Leptin, Estrogen, and Aromatase

Steroid hormone estrogen plays an important role in normal development as well as cancer progression [102]. Estrogen is primarily produced by aromatization of androstenedione via cytochrome P450 19A1 (CYP19A1) also known as aromatase in adipose tissues, which constitutes the primary source of estrogen in postmenopausal women [103]. Leptin is known to cross talk with estrogen signaling network to modulate estrogen receptor activation and increase the expression of ER-responsive genes. Leptin directly activates ERK phosphorylation which in turn induces phosphorylation of ER resulting in ER activation in a ligand-independent manner [43]. Interestingly, showing a functional dependence, leptin-mediated ER overexpression was blocked upon leptin-receptor silencing. Also, a strong correlation between leptin receptor and estrogen receptor has been reported in breast cancer samples from different stages of disease [101]. Leptin treatment increases ER expression in breast tumors in nude mouse xenograft model [104]. When breast cancer cells are treated chronically with leptin, it potentiates the proliferative actions of estrogen and alters the ratio of ERα to ERβ suggesting that elevated leptin levels in breast tumor microenvironment are capable of enhancing estrogen signaling [105]. Collectively, multiple studies have shown an important role of leptin in E2-ER network, increasing estrogen levels by increasing the conversion of androstenedione to estrogen and also activating ER function in a ligand-independent manner. As a positive feedback loop, estrogen also increases leptin function as estrogen receptor overexpression elevates leptin-induced Stat3 activity [106]. Leptin also increases aromatase expression and functional activity in an ERK and Stat3-dependent manner. Inhibition of ERK using MAPK inhibitor or overexpression of Stat3-dominant negative constructs blocks leptin-mediated aromatase expression [44]. Recently, leptin has been shown to influence estrogen metabolism in prostate cancer cells with an increase in ERα expression and decrease in the expression of ERβ. It is speculated that leptin's proliferative response in prostate cancer cells might be mediated partly by the alteration in estrogen metabolism [107]. Collectively, these studies show that there is a functional interaction between leptin and estrogen signaling networks.

Leptin, IGF1, Insulin, and EGFR

Elevated levels of both leptin and insulin are observed in obese state; therefore, it is interesting to note that a positive correlation between leptin and insulin exists in breast cancer cells as increased insulin levels also stimulates leptin expression [14, 108]. Increased level of IGF-1, a peptide growth factor that shares ~50 % sequence homology with insulin, is also observed in obese state [109]. Also, there is more than 50 % homology between insulin receptor (IR) and IGF-I receptors; as a result, both insulin and IGF-1 support interaction with IR and IGF-1R [110]. Interactions between leptin-signaling network and IGF-1 have been shown by our group and others [40, 111]. Our study shows a significant increase in proliferation as well as invasion and migration of breast cancer cells upon treatment with leptin and IGF-1. We report a novel bidirectional cross talk between leptin and IGF-1 signaling as IGF-1 results in the increased phosphorylation of leptin receptor (Ob-Rb) and leptin treatment induces tyrosine phosphorylation of IGF-1 receptor (IGF-1R). Co-treatment with leptin and IGF-1 results in synergistic phosphorylation of both Ob-R and IGF-1R. Concomitant activation of both Ob-Rb and IGF-1R results in activation of downstream effectors, Akt, ERK, IRS-1, and IRS-2. The signaling effects of Ob-R and IGF-1R converge on the transactivation of EGFR by proteolytic release of EGFR ligands as broad-spectrum matrix-metalloproteinase inhibitor GM6001 could inhibit this effect. These studies show that inhibition of EGFR activation using clinically relevant EGFR inhibitors, erlotinib, and lapatinib, can inhibit oncogenic signals of both leptin and IGF-1 [40]. Leptin has been shown to increase the proliferation of human esophageal adenocarcinoma cells by activating EGF-EGFR signaling axis [99]. Transactivation of EGFR is also involved in leptin-induced activation of JAK2 and ERK pathways in human gastric cancer cells. Leptin mediates EGFR activation in human gastric cancer cells via proteolytic release of EGFR ligand as a broad-spectrum matrix-metalloproteinase inhibitor GM6001 can inhibit leptin's effect [98].

Leptin, Notch, and Wnt/Catenin Pathway

The finding that Notch1 receptors act as survival factors promoting breast cancer has helped establish Notch1 signaling pathway as a pivotal target in breast cancer. Several studies have shown poor prognosis in cancer overexpressing leptin and Notch receptor. Our study shows an interesting mechanism by which leptin activates Notch1 receptor releasing transcriptionally active NICD which in turn binds to survivin promoter to increase survivin expression [31]. Specifically, this study shows the importance of leptin-induced survivin in migration of breast cancer cells. Importantly, inhibition of either EGFR or Notch1 results in blocking leptin-mediated cancer cell migration [31]. A cross talk between Notch, IL-6, and leptin has also been proposed to mediate cancer progression and metastasis which can be successfully inhibited using a γ-secretase inhibitor [100]. Leptin increases the expression of

genes involved in Wnt1-catenin pathway in colon epithelial cells [112]. Leptin induces EMT in breast cancer cells by activating Wnt1/catenin pathway which is regulated in part by activation of MTA1. Further elucidation of mechanistic underpinnings shows that leptin treatment increases the accumulation and translocation of β-catenin leading to increased binding to promoter. Upstream regulators of leptin's effect on β-catenin are MTA1 and Wnt1, whose activation leads to dissociation of destruction complex (GSK3β-LKB1-Axin-APC) and release of β-catenin. Silencing of MTA1 inhibits leptin-induced Wnt1 expression, GSK3 β phosphorylation, and β-catenin activation. Furthermore, MTA1 and β-catenin inhibition inhibits leptin-induced EMT and invasion and migration of breast cancer cells [41].

Collectively, these studies show multifaceted complex signaling network of leptin which mediates several biological functions of leptin. Understanding the molecular networks of leptin signaling and identifying critical molecules for biological functions of leptin can be extremely useful as this understanding can allow us to utilize existing, effective agents to inhibit highly active leptin network in breast cancer predictive and intervention purposes.

Strategies to Inhibit Leptin Signaling

Direct Inhibition of Leptin Signaling Using Small Molecule Inhibitors

Several strategies have been developed to block hyperactive leptin signaling, which might prove useful for cancer patients with elevated leptin levels. Elevated circulating levels of leptin have been reported in obese individuals. In addition, leptin and leptin receptor are overexpressed in many primary tumors as well as metastatic lesions. High levels of circulating leptin can be neutralized with soluble leptin receptors that can bind free leptin in circulation. Leptin receptors on tumor cells can be blocked by leptin antagonist that readily bind to leptin receptors and render them inactive by inhibiting intracellular signaling. Specific anti-leptin-receptor monoclonal antibodies (anti-LR mAbs) can be used to bind to the leptin receptor preventing leptin signaling. Anti-LR mAbs have a long half-life in the circulation because of their high molecular mass as well as good affinity for the receptor but these mouse-generated mAbs need to be humanized to eliminate their potential immunogenicity. Another effective approach is the development of recombinant, monomeric, small nanobodies to target leptin receptor (LR) and block the ligand-induced conformational change without interfering with the leptin-LR interaction. These nanobodies are stable and easy to manipulate [113, 114]. An important advantage of these nanobodies is that they do not cross blood-brain barrier, and therefore they can selectively inhibit peripheral activity of leptin. R128Q mutant of human leptin was the first leptin-receptor antagonist that showed activity in C57BL/6 and ob/ob mice [115]. L39A/D40A/F41A and L39A/D40A/F41A/I42A leptin muteins also show activity as potent competitive leptin-receptor antagonists [116]. Leptin activity can

also be antagonized by peptides derived from the leptin sequence. It has been reported that 26- and 32-aa-long peptides called LPA-2 and LP-1 can inhibit leptin action in vitro and in vivo at high doses [88, 117]. Importantly, recent development of superactive leptin muteins with antagonistic properties and other protein-blocking leptin activity present new possibilities for research [118].

Activation of Adiponectin as an Indirect Strategy to Inhibit Leptin

To date, there are several strategies to inhibit leptin and leptin receptor under various stages of development. An interesting approach to inhibit leptin signaling has stemmed from the fact that adiponectin, another adipocytokine, is negatively associated with obese state. Adiponectin is also called "the guardian angel adipocytokines" owing to its protective effects against many pathophysiological conditions. While most of the adipocytokines are causally linked to obesity-related diseases, adiponectin has shown promising insulin-sensitizing, anti-inflammatory, and anti-atherogenic activities. Decreased levels of adiponectin are observed in obesity and various obesity-related diseases. Several studies have shown the clinical relevance of adiponectin as treatment with adiponectin improves glucose/lipid homeostasis, increase insulin sensitivity, and prevent atherosclerosis in animal models [119–121]. These studies form the basis of research exploring the efficacy of adiponectin to inhibit oncogenic actions of leptin. Among several strategies to elevate adiponectin level, one effective strategy is the use of thiazolidinediones. The use of thiazolidinediones (TZDs) is associated with reduced cancer risk [122] and rosiglitazone, a TZD, is known to increase plasma adiponectin levels in overweight women with PCOS [123], type 2 diabetes mellitus patients, and patients with impaired glucose tolerance [124]. We examined whether rosiglitazone can be utilized to inhibit oncogenic actions of leptin. Breast cancer cells treated with rosiglitazone exhibited increased adiponectin levels and induced the activation of adiponectin-signaling network. Importantly, rosiglitazone could inhibit leptin-induced growth, invasion, and migration of breast cancer cells [35]. We show that adiponectin activates AMPK signaling pathway [125]. Metformin and honokiol, a bioactive molecule isolated from *Magnolia*, are reported to partially mimic adiponectin action and induce AMPK signaling in cancer cells to inhibit growth [126, 127]. Recent studies from our lab show that honokiol can effectively inhibit leptin-induced growth, EMT, and invasion and migration of breast cancer in vivo and in vitro models [128, 129]. Benzyl isothiocyanate treatment can also inhibit leptin signaling and its biological effects of breast cancer progression [130]. It is also known that exercise and loss of weight increase adiponectin levels which may protect against oncogenic effects of leptin. In fact, calorie-restricted mouse models and wheel running/exercise models do exhibit increased adiponectin levels and protection against carcinogenesis [131, 132] showing additional approaches to garner benefits from adiponectin. Several adiponectin-based peptide compounds acting as AdipoR (adiponectin receptor)

agonists are under preclinical development presenting other approaches for adiponectin-based therapeutics. Minimal adiponectin active site was identified to develop pharmacologically improved analogs. One such adiponectin receptor agonist is ADP 355 that can effectively inhibit growth of adiponectin receptor positive breast cancer cells (MCF7, MDA-MB-231) and alter adiponectin-signaling network [133]. While adiponectin analogues can be used to increase adiponectin levels, another strategy can be to modulate adipoR activity, augmentation of its effectiveness [134]. In summary, there are multiple approaches to activate/elevate adiponectin levels in order to achieve effective leptin inhibition.

Conclusion

Cancer progression is a multistep process involving various steps such as tumor initiation, primary tumor growth, invasion, and metastatic progression which involves complex interaction with various stromal components including endothelial cells, immune cells, fibroblasts, and adipocytes. Studies over the past few years have clearly shown that leptin affects cancer incidence, growth, and metastatic progression. Leptin cross talks with several other oncogenic pathways in cancer cells and forms a hyperactive leptin-signaling network. Understanding the cross talk of leptin with other signaling pathways has given us the opportunity to develop multiple ways to inhibit key signaling nodes to inhibit oncogenic actions of leptin. While there are several small molecules, chemical inhibitors, adipocytokines-based, and bioactive strategies to inhibit oncogenic actions of leptin, additional research is needed to standardize leptin inhibition strategies for clinical purposes.

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Chapter 5
Retinol Binding Protein 4: Role in Diabetes and Cancer

Daniel C. Berry and Noa Noy

Abstract Vitamin A, retinol, circulates in blood associated with retinol-binding protein 4 (RBP4). It was reported that the level of circulating RBP4 is often elevated in obese mice and humans and that, under these circumstances, the protein induces insulin resistance. Recent studies showed that, in addition to its function as a transport protein, RBP4 serves as a cytokine which, upon binding to a cognate membrane receptor termed STRA6, activates a signaling cascade mediated by the Janus kinase JAK2 and its associated transcription factors STAT3 and STAT5. In turn, activated STATs induce the expression of target genes, some of which are closely involved in the regulation of insulin responsiveness and cancer cell biology. Taken together, available information suggests that through its unexpected activity as a signaling molecule, RBP4 may be a link through which obesity results in insulin resistance and some cancers.

Keywords Vitamin A • Retinol-binding protein • Cytokine • Insulin responses • Cancer • JAK • STAT • Intracellular retinoid-binding proteins

Vitamin A: Absorption and Metabolism

Vitamin A, retinol, is an essential lipid-soluble factor, which can be obtained from two major dietary sources: animal fat/fish oil and plant material [1, 2]. Retinyl esters, present in animal fat and fish oil, are hydrolyzed in the intestinal lumen to yield free fatty acid and retinol (Fig. 5.1) which is then subsequently taken up by the enterocytes. Carotenoids, present in plant material, are absorbed intact, and, in the enterocyte and other tissues, they can undergo enzymatic cleavage into two

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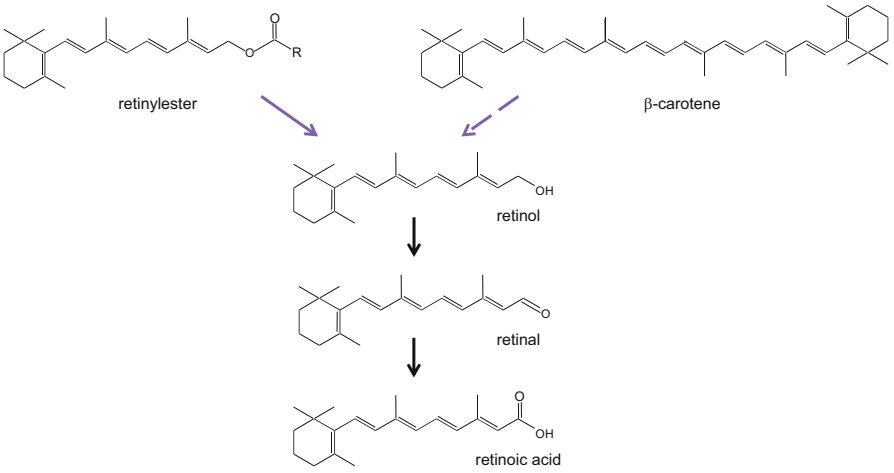

Fig. 5.1 Structures of retinol and its precursors and active metabolites

molecules of retinal which is then converted to retinol (Fig. 5.1). Vitamin A is essential for vision, reproduction, embryonic development, immune function, and tissue homeostasis.

In the enterocytes, retinol, obtained from either dietary food source, is esterified with a long chain fatty acid to yield retinyl esters. These are packaged in chylomicrons and secreted into the lymphatic system to be delivered through the circulation to tissues that store or utilize vitamin A. Two types of enzymes catalyze the formation of retinyl esters [3, 4]. One of these is lecithin/retinol acyltransferase (LRAT), an enzyme that synthesizes retinyl esters by catalyzing the transesterification of a fatty acyl moiety from the sn-1 position of phosphatidylcholine to retinol. The other type of enzyme, termed acyl coenzyme A/retinol acyltransferase (ARAT), utilizes activated fatty acids in the form of fatty acyl-CoAs to form retinyl esters. The enzyme diacylglycerol acyltransferase 1 (DGAT1), long known to catalyze the formation of triglycerides [5], can also utilize retinol as a substrate thereby functioning as an ARAT to form retinyl esters [6]. Both ARAT and LRAT are integral endoplasmic reticulum membrane proteins that are present in various tissues [7]. LRAT is predominantly important for retinyl ester formation in the liver, testes, retina, and other tissues, while DGAT1 plays a key role in retinol esterification in the lactating mammary gland, skin, and adipose tissue [8]. The relative contribution of the two enzymes to retinyl ester synthesis thus appears to be tissue-specific.

The major site of vitamin A storage in the body is the liver [9]. Following uptake of retinyl esters from chylomicrons into hepatic cells, vitamin A is transferred to hepatic stellate cells where retinyl esters are stored in lipid droplets [9]. The mechanism by which vitamin A moves between hepatocytes and stellate cells is incompletely understood, but available information suggests that it is transported between the two cell types in the form of free retinol [10]. Absorption and mobilization of vitamin A between different tissues and cells thus require continuous hydrolysis

and reformation of retinyl esters. Notably, in addition to the liver, vitamin A is stored in various other tissues including the adipose depots, kidney, testis, lung, bone marrow, and eye.

It has been long established that many of the biological activities of vitamin A are exerted by its transcriptionally active metabolite retinoic acid (RA). RA is produced from retinol by two sequential oxidation steps: retinol is converted to retinal, which is then oxidized into RA (Fig. 5.1). These metabolic conversions are catalyzed respectively by retinol dehydrogenases and retinal dehydrogenases in reactions that entail the dehydrogenation of the substrates using the electron acceptors NAD^+ or $NADP^+$. Two classes of enzymes can function as retinol dehydrogenases (RolDH) in vitro. Cytosolic medium-chain alcohol dehydrogenases and members of the family of short-chain dehydrogenases/reductases (SDRs) are associated with endoplasmic reticulum. In contrast with soluble alcohol dehydrogenases, some SDRs can metabolize retinol when bound to the cellular retinol-binding protein CRBP1. Hence, considering that the major fraction of retinol in cells is bound to a CRBP (see below), it is currently believed that retinal formation in vivo occurs mainly by SDRs [11, 12].

The irreversible second step in RA synthesis is catalyzed by retinal dehydrogenases (RalDH). To date, four mammalian RalDHs have been identified. Genetic ablation of RalDH2 is embryonic lethal by day E9.5 [13]. RalDH1-null mice are viable and exhibit no gross malformations, but liver RA synthesis is greatly reduced [14]. Mice lacking RalDH3 are impaired in RA synthesis and display ocular and nasal malformations similar to those observed in vitamin A-deficient fetuses [15]. Less is known about the physiological function of RalDH4 which displays enzymatic selectivity toward 9-*cis*-retinal and may play a role in synthesis of 9-*cis*-retinoic acid [16].

Retinoid-Binding Proteins

Due to their hydrophobic nature, retinoids efficiently dissolve into the hydrophobic core of cellular membranes but do not readily traverse aqueous spaces such as plasma and cytosol. Extra- and intracellular trafficking of these compounds thus requires the presence of water-soluble proteins that specifically bind different retinoids and shuttle them to their sites of metabolism and action. In blood, retinol circulates bound to serum retinol-binding protein (RBP, encoded for by the *Rbp4* gene). In cells, there exist proteins that selectively bind retinol and retinal or RA. Cellular retinol- and RA-binding proteins (CRBP, CRABP) are highly conserved across species that utilize vitamin A, implying that they play critical roles in vitamin A biology. Retinoid-binding proteins solubilize their ligands in aqueous spaces, protect them from nonspecific degradation, and protect cellular membranes from disruption by the amphipathic retinoids. Retinoid-binding proteins also regulate the biological activities of their respective ligands by selectively delivering them to specific sites of metabolism and action.

RBP4 and Transport of Retinol in the Circulation

Retinol circulates in blood bound to RBP4, a 21,000 Da protein that belongs to the lipocalin family of proteins and that contains one binding site for retinol. Various tissues, including adipose, kidney, testis, brain, and digestive tract tissues, can synthesize and secret RBP4, but the main source of this protein under normal physiological circumstances is the liver. Secretion of RBP4 from the liver is tightly regulated by the availability of retinol [17]. During vitamin A deficiency, RBP4 secretion is inhibited, and the protein accumulates in endoplasmic reticulum. Upon increase in retinol levels, RBP4 moves to the Golgi and is secreted into blood in the form of the holo-protein. In blood, RBP4 is bound to another protein called transthyretin (TTR), a 56,000 Da homotetramer that, in addition to associating with RBP, functions as a carrier for thyroid hormones. Retinol bound in a ternary TTR-RBP-retinol complex thus circulates. It is believed that binding of RBP4 to TTR serves to prevent the loss of the smaller protein from the circulation by filtration in the glomeruli of the kidney. The concentration of the holo-RBP-TTR complex in plasma is usually kept at ~1–2 μM, but blood concentrations of holo-RBP4 and TTR and thus the stoichiometry of the complex can vary under various conditions, e.g., in response to vitamin A deficiency and acute stress or in pathologies such as obesity. As retinal and RA differ from retinol only by the composition of their head groups, RBP4 displays a broad specificity for retinoids. However, in the presence of ligands with polar end groups larger than a hydroxyl, such as the carboxyl group of retinoic acid, the interactions of RBP4 with TTR are hindered [18]. Consequently, the only retinoid that is found to be associated with RBP in plasma is retinol.

Cellular Retinol-Binding Proteins

Cellular retinol-binding proteins (CRBP) belong to the family of small (~15,000 Da) intracellular lipid-binding proteins (iLBP) whose members include proteins that bind various lipophilic ligands. The iLBPs share a β-clam structure comprised of two 5-stranded orthogonal β-sheets that form a ligand-binding pocket. A helix-loop-helix forms a "lid" over the entrance to the ligand-binding pocket of these proteins [19–21]. Although similar in their three-dimensional structures, iLBPs are not as homologous in their primary sequences, and they bind lipophilic molecules with distinct selectivity. Of note, CRBPs and RBP4 display distinct modes in association with their ligand. Hence, retinol binds to RBP4 with the β-ionone ring buried deep in the binding pocket and the alcohol moiety located at the entrance to the binding pocket, but it associates with CRBPs in the reverse orientation.

Four isotypes of CRBPs (CRBP1-CRBP4) have been identified to date, and it has long been suggested that these proteins function to deliver retinol to specific enzymes. It was thus shown that CRBP1 delivers retinol to LRAT, and, indeed,

CRBP1 deficiency is accompanied by a 50 % reduction of retinyl ester pools in hepatic stellate cells [22]. A question that arises from the observations that retinol bound to CRBPs can serve as substrate for particular enzymes relates to the mechanism by which the enzymes access this ligand. This question is especially intriguing as retinol is bound to CRBP with the polar end group buried deeply inside the binding pocket [23]. It is thus difficult to envision how any enzyme could gain access to the ligand's end group in such a location. Possibly, protein-protein interactions between the binding protein and specific enzymes result in a conformational change in CRBP, allowing for direct movement of retinol from the binding protein into the enzyme's active site.

Vitamin A Uptake by Extrahepatic Tissues: RBP4 and STRA6

The mechanism by which retinol leaves circulating RBP4 and is taken up by target cells has long been controversial. Early biochemical studies showed that the association between retinol and RBP4 is reversible and that retinol can spontaneously dissociate from RBP4 or the RBP4-TTR complex with a $t_{1/2}$ of minutes [24–26]. Further, these studies demonstrated that retinol rapidly crosses lipid bilayers and diffuses from the membrane/lipid environment within seconds [27]. These observations indicated that retinol can leave its serum carrier and move into cells by free diffusion at rates that are sufficient for physiological needs. Studies of the kinetics of uptake and metabolism of retinol in keratinocytes supported this conclusion. These studies showed that the rate of entry of retinol is similar regardless of whether it is provided to the cells in a free or RBP4-bound form [28], suggesting that the rate of entry of retinol into cells is dictated by its concentration and metabolism and is not limited by the rate of its uptake at the plasma membrane. On the other hand, it has been proposed that a specific receptor exists which recognizes circulating RBP4 and mediates retinol transfer from the binding protein into cells [29, 30].

In 2007, a hallmark study demonstrated that an RBP4 receptor exists in some non-hepatic tissues [31]. The gene encoding this protein was previously identified in embryonic carcinoma cells to be a RA-responsive gene, and it was consequently termed stimulated by retinoic acid 6 (STRA6) [32]. In the adult, STRA6 is most highly expressed in retinal pigment epithelium (RPE) in the eye; however, STRA6 is also expressed in many other tissues including the brain, adipose tissue, spleen, kidney, testis, and female genital tract but is undetectable in the liver and intestines [31–33]. The ontology of STRA6 is unclear because it lacks homology to any known protein, but this gene is conserved from zebra fish to mammals [31]. The STRA6 gene encodes a 74 Kd protein that is largely hydrophobic. Computer modeling and mutagenesis analyses respectively suggested that STRA6 could be arranged in 11 or 9 transmembrane helices, a number of loops, and a large cytosolic C-terminus tail [34–36]. To date, the three-dimensional structure of the protein, conclusively demonstrating the orientation of the protein within the membrane, has not been solved. Importantly, retinol uptake by STRA6 is not affected by metabolic

inhibitors [31], indicating that receptor is not energy-driven rather that it mediates facilitated diffusion of retinol. Retinol thus enters cells following an inwardly directed concentration gradient.

It has been reported that human mutations in the STRA6 gene are associated with the occurrence of Matthew-Wood syndrome, a severe congenital disease that includes microphthalmia, pulmonary hypoplasia, heart defects, and diaphragmatic hernia [37–39]. These observations led to the suggestion that STRA6 is critically involved in regulating vitamin A homeostasis. However, in contrast with mutations in STRA6, human mutations in RBP4 are associated with only mild symptoms traceable to vitamin A deficiency, including night blindness and a modest retinal dystrophy [40]. It was recently reported that missense mutations in RBP4 that are associated with eye defects decrease the retinol-binding affinity of RBP4 but increase the affinity of the protein for STRA6. Mutant RBP4 thus blocks the ability of WT-RBP4 to access STRA6 [41]. The lack of phenotypic relationship between humans with STRA6 and RBP4 mutations suggests that STRA6, in addition to mediating retinol uptake from RBP4, may have other biological functions. Alternatively, Matthew-Wood syndrome may originate from more extensive chromosomal aberrations other than STRA6 mutations. In support of this notion, it has been shown that a patient suffering from PAGOD syndrome, a multiple congenital anomalies with significant phenotypic overlap with Matthew-Wood syndrome, does not carry any STRA6 mutations [42]. Additional information is needed to better understand the molecular basis for Matthew-Wood syndrome.

STRA6-null mice are obtained at Mendelian frequency, appear healthy, and do not display any histological abnormality except in the eye [33, 43–46 and see http://www.kompphenotype.org/summary-tab.php?gene=STRA6]. These observations indicate that STRA6 is dispensable for maintaining cellular retinol uptake in most tissues during embryonic development and in the adult. The exception is the eye where STRA6 deficiency leads to a reduction in retinol uptake, causing severe depletion of retinoid stores in the retinal pigment epithelium (RPE) and neurosensory retina [43, 45]. Morphologically, the eyes of STRA6-null mice display a shortened rod outer and inner segments and a reduction in cone photoreceptor cell number and cone b-wave amplitude [44]. The eyes of STRA6-null mice are also characterized by the presence of vascularized structures in the vitreous humor between the posterior surface of the lens and neurosensory retina. However, these morphological changes and reduction in visual function are mild, suggesting that retinol can enter the RPE by non-STRA6-mediated pathway(s) [44]. Examination of other STRA6-expressing tissues showed that the rate of retinol uptake from circulating holo-RBP4 is only modestly reduced in STRA6-null mice. Consistent with normal retinol uptake, retinoid content of non-hepatic tissues is indistinguishable between STRA6-null and WT mice fed either a vitamin A-sufficient or a vitamin A-deficient diet [33]. Moreover, STRA6-null fetuses born from dams fed a vitamin A-deficient diet throughout pregnancy do not exhibit any hallmarks of fetal vitamin A deficiency syndrome [33]. These observations demonstrate that, although STRA6 functions as a retinol transporter, the major fraction of cellular uptake of retinol from circulating holo-RBP4 occurs in a STRA6-independent fashion, likely by free

diffusion across the plasma membrane [18, 47]. In the eye, diffusion-mediated retinol uptake is supplemented by STRA6-mediated transport to satisfy the extreme vitamin A demand of this tissue.

The identification of STRA6 as a retinol transporter raises interesting questions regarding the mechanism by which this protein facilitates vitamin A uptake. It has been reported that a functional cooperation exists between STRA6 and lecithin/retinol acyltransferase (LRAT), which catalyzes the conversion of retinol into its storage form, retinyl esters [48, 49]. However, the subcellular localization of LRAT renders it unlikely that the enzyme can receive the hydrophobic retinol directly from the plasma membrane-bound STRA6. Thus, scientific focus turned to intracellular retinol-binding proteins such as CRBP1, which binds retinol and shuttles it to different cellular destinations. Early studies demonstrated that CRBP1 facilitates STRA6-mediated retinol transport into the cell but did not delineate the mechanism that may underlie such cooperation [50]. Our studies showed that CRBP1 physically interacts with STRA6 to directly receive retinol entering the cell through the receptor [51]. Specifically, we found that apo-CRBP1 directly binds to STRA6 through an intracellular loop of the receptor, which we termed the CRBP-binding loop (CBL), as well as through a region in the receptor cytoplasmic C-terminus [51] (Fig. 5.2). We further showed that CRBP1 dissociates from STRA6 following its ligation by retinol. The "switch" that allows retinol to control the association of CRBP1 with the STRA6-CBL was mapped to R22, R31, and K32 at the helix-loop-helix that caps that entrance to CRBP1's ligand-binding pocket [51]. Notably, these residues are conserved among several intracellular lipid-binding proteins (iLBPs), but they are used for different purposes [51]. In the iLBP family, cellular RA-binding protein 2 (CRABP2), and fatty acid-binding proteins 4 and 5 (FABP4, FABP5), these residues comprise a ligand-controlled nuclear localization signal [52–54]. In contrast, in CRBP1, these residues mediate the interaction of this protein with STRA6, and their mutation impairs both the association of the binding protein with the receptor and STRA6-mediated vitamin A uptake [51]. Hence, STRA6-mediates direct transport of retinol from extracellular RBP4 to intracellular CRBP1.

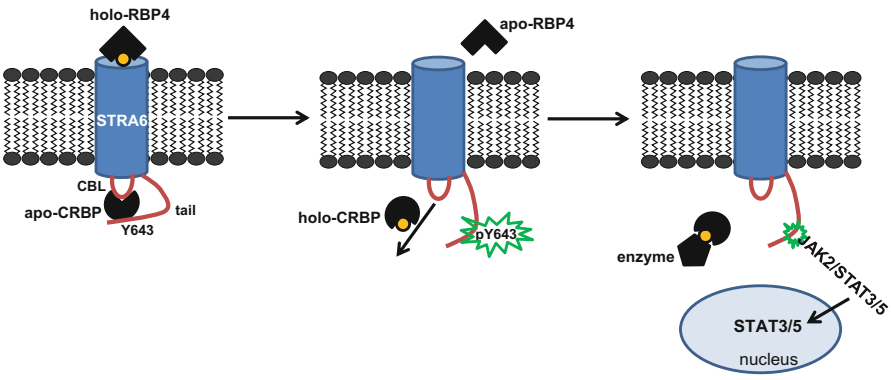

Fig. 5.2 Model for the mechanism of action of the RBP4/STRA6 cascade. See text

Following dissociation from STRA6, CRBP1 delivers retinol to LRAT (Fig. 5.2). The mechanism through which retinol is transferred from the binding protein to the enzyme has not been directly investigated, but it has been suggested that it entails interactions between the two proteins, enabling direct substrate channeling [22, 55]. Retinol thus transfers from RBP4 through STRA6 to CRBP1 and to LRAT, bypassing the need for the hydrophobic vitamin to move through an aqueous phase. These observations provide a clear explanation for the observed functional cooperation between STRA6 and LRAT. Specifically, the data show that LRAT supports STRA6-mediated retinol uptake both by metabolizing retinol to generate inwardly directed retinol concentration gradient and by unloading retinol from CRBP1, allowing the binding protein to reassociate with STRA6 and thus maintain retinol transport [49, 51].

It has been suggested that STRA6 not only transports retinol from blood into cells but also mediates retinol efflux from cells upon addition of extracellular apo-RBP4 [56, 57]. However, several lines of evidence render such an activity highly unlikely: (1) In cells, retinol is bound to CRBPs. As STRA6 binds apo-CRBP but does not associate with retinol-bound CRBP [51, 58], the receptor cannot access intracellular retinol. (2) STRA6 binds extracellular holo-RBP4 but does not associate with apo-RBP4 [41, 59]. It thus cannot mediate efflux onto apo-RBP4. (3) RBP4-bound retinol circulates at 1–2 μM, a higher level than that of intracellular free retinol. As STRA6 mediates facilitated diffusion and not active transport, the receptor is unable to mediate transport against a concentration gradient [31, 60]. (4) If STRA6 is involved in efflux, one might expect that STRA6-null mice will display lower serum levels and higher concentration of vitamin A in tissues. However, these mice display normal retinol levels in both serum and most tissues [33].

RBP4 Level Is Correlated with Obesity and Insulin Resistance

The adipose tissue is central for regulating obesity and insulin responses, and adipocytes hold clues critical for understanding metabolic dysfunction [61]. The retinol metabolite retinoic acid decreases adiposity, increases lipid oxidation, enhances insulin sensitivity, and blunts adipogenesis [62–66]. In sharp contrast to data demonstrating the beneficial effects of retinoic acid against adiposity and diabetes, it was surprisingly shown that circulating level of the vitamin A carrier RBP4 is elevated in obese and diabetic rodents and humans and that the protein causes insulin resistance in rodents [67]. Subsequent studies showed that serum RBP4 levels often correlate with weight and glucose sensitivity, waist-to-hip circumference ratio, and waist circumference and percent trunk fat, all of which are indicators of metabolic dysfunction and obesity [68–71]. Visceral fat is thought to promote insulin resistance and exacerbate metabolic dysfunction [72]. Interestingly, RBP4 mRNA expression was found to be 60-fold higher in visceral fat of obese subjects compared to lean individual [70]. These studies showed that adipose RBP4 expression positively correlates with intra-abdominal fat mass and inversely with insulin

sensitivity. Obesogenic upregulation of RBP4 has not been consistently observed in obese or diabetic patients (see [73] for review). Nevertheless, it has been reported that obese patients that have undergone weight reduction display decreased RBP4 expression [74], and several studies documented that reducing serum RBP4 levels may lead to therapeutic benefit [75–78].

Notably, while RBP4 causes insulin resistance, association with its blood binding partner TTR negates this activity [58]. Hence, while mice injected with RBP4 develop insulin resistance, mice injected with RBP4 complexed with TTR do not [58]. These data established that RBP4 suppresses insulin signaling only when its blood level exceeds that of TTR. Indeed, while adipose RBP4 expression is upregulated in obese animals, TTR expression in the liver, adipose tissue, and serum are unaltered in response to caloric excess, fat mass, and insulin sensitivity [58, 76]. Alterations of the RBP4/TTR ratio in blood could emanate from the transcriptional regulation of these two genes. TTR is predominantly expressed in two tissue types: the central nervous system and the liver with the latter comprising the predominant source for the protein in the serum [79]. Indeed, in response to inflammation, expression of hepatic TTR is downregulated, and, consequently, serum level of TTR dramatically decreases [80, 81]. Moreover, it has been reported that sex hormones regulate hepatic TTR expression and, in turn, control TTR serum levels. Dysregulation of sex hormone levels in obesity and cancer [82] may thus contribute to alteration in serum RBP4/TTR ratio.

In addition to protecting against RBP4-induced insulin resistance, an important function of TTR is to protect RBP4 from filtration by the kidney, and disruption of the RBP4/TTR complex leads to loss of RBP4 from blood. It has thus been suggested that usage of compounds that disrupt the RBP4/TTR complex as fenretinide may comprise a pharmacological approach for reducing serum RBP4 levels [9, 60, 83]. In addition to disrupting RBP4/TTR interaction, it has been proposed that fenretinide transcriptionally represses RBP4 expression [84, 85] thereby further reducing serum RBP4 concentrations. Notably, however, fenretinide appears to also have RBP4-independent actions on adiposity; for instance, fenretinide induced similar weight loss in RBP4-null and in WT mice fed a high-fat diet [86]. Thus, the mechanism(s) through which fenretinide functions in obese and diabetic patients remains poorly understood.

RBP4 and STRA6 in Insulin Resistance: A Mechanism of Action

The observation that RBP4 causes insulin resistance raises questions regarding the mechanism by which the protein exerts such an unexpected activity. The observation that injecting RBP4 into lean mice was sufficient to induce insulin resistance implies that circulating RBP4 rather than intracellular RBP4 controls insulin sensitivity [67] and raises the possibility that the protein functions as a cytokine which is recognized by a receptor which, in turn, triggers a signaling cascade. The RBP4

receptor STRA6 was indeed found to function in this fashion [87]. Inspection of the primary sequence of STRA6 revealed that its cytosolic domain contains a putative phosphotyrosine motif [39, 87]. A series of biochemical and molecular studies showed that the putative phosphotyrosine residue of STRA6 is phosphorylated upon association of the receptor with holo-RBP4 and the ensuing retinol transport [87] (Fig. 5.2). Phosphorylation of this site leads to the recruitment of Janus kinase 2 (JAK2) thereby recruiting and activating the transcription factors signal transducer and activator of transcription (STAT) 5 or, in a cell-dependent manner, STAT3 [87, 88]. Activated STATs then translocate to the nucleus and regulate expression of a host of STAT target genes [89] (Fig. 5.2). Importantly, STAT target genes in adipose tissue and muscle include suppressors of cytokine signaling (SOCS), genes that encode proteins that potently suppress the activity of multiple cytokine receptors, including that of the insulin receptor [90, 91].

It has been demonstrated that treatment of cultured adipocytes with RBP4 triggers phosphorylation of STRA6, activation of JAK2 and STAT5, and upregulation of SOCS3, leading to suppression of insulin responses [51, 58, 87]. It was further shown that neither apo-RBP4 nor retinol exerts such an activity and that the path is not activated by retinoic acid [87]. Similarly, injection of RBP4 into mice resulted in activation of STRA6, leading to phosphorylation of JAK2 and STAT5 and to subsequent upregulation of SOCS3 in adipose tissue and muscle [33, 51, 58, 87]. Consequently, administration of RBP4 decreased the phosphorylation status of both IR and its downstream effector Akt1 in these tissues. Supporting the STRA6 specificity of the response, RBP4 did not affect JAK/STAT activation, the level of SOCS3, or the phosphorylation status of IR in the liver, a tissue that does not express the receptor, or in STRA6-null mice [33, 51, 58, 87]. Further evidence for the critical role of STRA6 in suppression of insulin signaling by RBP4 was provided by the observations that mice lacking STRA6 or the intracellular components that enable STRA6 function, namely, CRBP1 and LRAT, are protected from insulin resistance brought about by the administration of RBP4 [33, 49]. Moreover, STRA6-null mice fed a high-fat diet remain glucose sensitive in the presence of elevated serum RBP4 levels [33], and even partial reduction in STRA6 expression only in adipose tissue protects mice from high-fat feeding-induced insulin resistance [92]. Collectively, these studies show that holo-RBP4 functions like a classical cytokine to activate a STRA6/JAK2/STAT3/5 pathway. However, STRA6 appears to be unique among known cytokine receptors in that it functions both as a transporter and as a signaling receptor.

It is important to note that the two functions of this “cytokine signaling transporter” are strictly interdependent, i.e., STRA6 signaling critically depends on STRA6-mediated retinol transport and, vice versa, retinol transport cannot proceed if STRA6 phosphorylation is impaired [33, 51]. Hence, treatment of cells with a JAK2 inhibitor or mutation of the STRA6 phosphotyrosine motif inhibit STRA6-mediated vitamin A transport [51]. Moreover, like retinol transport, STRA6-mediated cell signaling requires expression of the intracellular accessory proteins CRBP1 [51] and LRAT; retinol-dependent association of CRBP1 with the receptor as well as expression of LRAT is required for enabling STRA6 phosphorylation [49]. Whether CRBPs other than CRBP1 and retinol-metabolizing enzymes other than LRAT can support the activation of STRA6 remains to be investigated.

Alternative Models for Understanding How RBP4 Induces Insulin Resistance

In diabetes and obesity (diabesity) and other metabolic dysfunctional states, adipose tissues undergo an array of assaults and alterations including hypertrophy of existing adipocytes, deprivation of oxygen (hypoxia), vascular remodeling, adipose hyperplasia, and adipose tissue inflammation [61, 93]. Obesity-induced upregulation of RBP4 in adipose tissue may thus lead to deleterious metabolic effects by mechanisms other than activation of STRA6. Alternative models have been put forth, but all seem to echo that RBP4 elicits some sort of a signaling event. For example, it was reported that treatment of adipocytes with RBP4 blocked phosphorylation of the insulin receptor substrate 1 in response to insulin receptor activation [94]. Whether this reflects an RBP4-induced STRA6-mediated event or a STRA6-independent activity was not explored. Another study proposed that RBP4 induces expression of proinflammatory cytokines by directly activating adipose tissue antigen-presenting cells (APC) through activation of the JNK pathway which, in turn, induces CD206+ macrophages to secrete proinflammatory cytokines [95]. Moreover stimulation of APCs by RBP4 was found to be sufficient to cause insulin resistance in a murine model. RBP4 was also shown to activate JNK in adipose tissue-derived macrophages, and this activity appears to be mediated by toll-like receptor 4 (TLR4) [95, 96]. Stimulation of this pathway by RBP4 promoted inflammatory responses by upregulating genes such as IL-6, TNFa, and MCP1, and coculturing RBP4-stimulated macrophages with adipocytes induced insulin resistance [96]. These manuscripts suggested that both apo-RBP4 and holo-RBP4 can activate macrophage TLR4 signaling [95, 96]. A recent study also purposed that RBP4 and STRA6 cooperate to control adipocyte differentiation and that they do so not by direct signaling but by controlling the cellular production of retinoic acid [97]. It was thus reported that holo-RBP4 increased the retinoid content of preadipocytes, thereby activating the RA receptor RARα, which inhibits differentiation. Conversely, apo-RBP4 decreased progenitor intracellular retinoid levels, allowing adipogenesis to occur [97]. As scientific progress continues, it will be exciting to find out whether and how pathways other than the one mediated by STRA6 may be involved in the ability of RBP4 to control adipose tissue biology, metabolism, insulin responses, and energy homeostasis.

Involvement of RBP4 and STRA6 in Cancer Cell Biology

Obesity and metabolic dysfunction is a primary culprit in promoting several cancer types, including breast, colon, and pancreatic cancers [98, 99]. The observation that RBP4 is upregulated in obese animals and that it triggers STRA6-mediated activation of STAT3, a known oncogene [100], suggests that the path may play an important role in promoting obesity-associated cancers. Indeed, STRA6 has been shown to be upregulated in several cancers, including ovarian and endometrium cancers,

triple-negative breast cancer, Wilms' kidney tumors, melanomas, and colon polyps and colorectal tumors [101]. For example, while not expressed in normal colon or normal mammary epithelium, the expression of both STRA6 and RBP4 is upregulated early in the development of colon and breast cancers, and the high level of expression is sustained throughout the progression of these diseases [88, 101]. Similarly, STRA6 was found to be upregulated in APC^{min} mice, a rodent model for human colorectal adenomas [88]. Moreover, epidemiological studies have shown that increased serum RBP4 level correlates with specific types of cancer including colon, pancreatic, and breast cancers [73, 102, 103]. Serum RBP4 levels also correlate with polycystic ovary syndrome (PCOS), a syndrome that displays a range of symptoms that stem from hormonal imbalance [104]. Development of PCOS triples women's risk of developing ovarian cancer. Notably, the severity of PCOS is increased in women who are obese [104]. Taken together, genetic and epidemiological studies suggest that the RBP4/STRA6 pathway may comprise an important link between metabolic diseases and some cancers.

While much of the how and when RBP4 and STRA6 are upregulated in specific tumors remains unexplored, recent studies established that the pathway is highly oncogenic. Hence, experiments using cultured mammary and colorectal carcinoma cell lines showed that expression of STRA6 and RBP4 positively correlates with the level of malignancy and tumor aggressiveness [88]. Additional studies showed that downregulating STRA6 in highly tumorigenic mammary and colorectal carcinoma cell lines suppressed oncogenic activities such as proliferation, migration, invasion, and colony formation [88]. Conversely, upregulating STRA6 expression in less aggressive cell lines drove oncogenic properties. Remarkably, addition of RBP4 to culture media stimulated the ability of STRA6 to promote oncogenic activities. In vivo studies using xenograft mouse models of colorectal cancer demonstrated that tumor growth was significantly suppressed upon downregulation of STRA6 expression and, conversely, overexpressing STRA6 facilitated tumor formation and markedly enhanced tumor growth [88].

Biochemical and molecular studies showed that RBP4/STRA6 stimulated JAK2 phosphorylation and activation in both mammary and colorectal carcinoma cell lines. Interestingly, while STRA6 activates STAT5 in adipose tissue and skeletal muscle, it predominantly triggers the activation of the highly oncogenic STAT3 in these cancer cells. Molecular analysis showed that RBP4/STRA6 signaling culminated in transcriptional upregulation of classical STAT oncogenic target genes such as c-Fos, cyclin D1, VEGF-A, and matrix metallopeptidase 9 (MMP9) [88]. To ascertain if RBP4/STRA6 pathway could initiate cell transformation, the classical non-transformed NIH-3T3 fibroblasts were used to assess whether the cascade can initiate tumor development. NIH-3T3 cells express CRBP1 but do not express STRA6 or any examined retinol-metabolizing enzymes. Overexpression of STRA6 alone was not sufficient to induce oncogenic transformation in these cells, but ectopic co-expression of STRA6 together with its enabling enzyme LRAT promoted cell proliferation, migration, and contact-independent growth, iconic hallmarks of oncogenic transformation [88]. In contrast, overexpression of LRAT with a STRA6 mutant lacking the phosphotyrosine motif did not promote oncogenic activities, supporting the importance of STRA6 signaling in this activity. Moreover, NIH-3T3

cells ectopically expressing LRAT and STRA6, but not LRAT together with a signaling-defective STRA6 mutant, effectively formed tumors in a xenograft mouse model [88]. Hence, while these are early days in understanding the role of RBP4/STRA6 in cancer biology, available information established that the signaling cascade initiated by RBP4 and STRA6 is a bona fide driver which not only promotes but initiates oncogenic transformation.

Therapeutic Relevance

We are only at the initial phase of understanding the complete spectrum of the biological roles and mechanisms of action of RBP4 and STRA6. However, available information established that these two proteins cooperate to regulate multiple biological functions including adipogenesis, lipid and sugar homeostasis, insulin signaling, oncogenic transformation, and cancer cell growth. The RBP4/STRA6 cascade thus appears to comprise a druggable target for novel therapeutic approaches for metabolic diseases as well as some cancers. As noted above, it has been proposed that approaches that target the removal of RBP4 from blood may be used for ameliorating insulin resistance [86]. However, a better therapeutic approach may lie in targeting the RBP4/STRA6 path [105]. For example, biochemical studies have shown that CRBP1 interacts with STRA6 and the STRA6 residues that mediate this interaction have been identified [51]. A peptide that could block this interaction could be beneficial for inhibiting STRA6-mediated signaling. Further, RBP4 ligands that are nontransferable to CRBP1 may block STRA6-mediated movement of retinol from RBP4 to CRBP1, thereby blunting STRA6 signaling [51]. Genetic approaches to targeting the pathway may also be beneficial. Information on the gene networks that regulate the expression of components of the path may be used to gain insight into how expression levels of these proteins become altered and provide diagnostic markers. Furthermore, understanding the basis for the observations that RBP4 is upregulated in some but not in all obese and diabetic patients [106–108] would be useful for devising novel strategies for therapy and perhaps prevention of disease. Finally, detailed structure-function information on STRA6 will help elucidate potential binding sites of other proteins and enable directed design of strategies to interfere with the association of the receptor with its accessory proteins.

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Chapter 6
Visfatin, Obesity, and Cancer

Maria Dalamaga and Gerasimos Socrates Christodoulatos

Abstract Cancer represents an important public health concern. Overweight, obesity, and metabolic syndrome influence the risk and prognosis of several disease states including cancer. Metabolic changes related to visceral obesity could contribute to a dysfunctional adipose tissue provoking chronic subclinical inflammation, insulin resistance, and abnormal production of adipokines. Visfatin, found in the visceral adipose tissue, known also as nicotinamide phosphoribosyltransferase (Nampt) and pre-B-cell colony-enhancing factor (PBEF), acts as a multifaceted molecule with a triple action: a cytokine, a growth factor, and an enzyme. It exerts a pivotal role in a multitude of metabolic and stress responses and cellular bioenergetics, specifically nicotinamide adenine dinucleotide (NAD) synthesis. Visfatin/Nampt exhibits antiapoptotic, proliferative, pro-inflammatory, pro-angiogenic, and metastatic properties. The insulin-mimetic function of visfatin/Nampt remains a controversial issue. Circulating visfatin/Nampt is enhanced in many cancers, including obesity-associated malignancies. It is associated with bad prognosis and higher tumor stage and grade. Plasma visfatin/Nampt may be a novel risk factor as well as a surrogate clinical marker in cancer therapeutics. Moreover, pharmacologic neutralization of visfatin/Nampt employing agents that reduce its levels or downregulate signaling pathways downstream of visfatin/Nampt could be promising anticancer treatments. In this book chapter, we will particularly focus on both intracellular and extracellular visfatin/Nampt's contribution to cancer pathophysiology as well as on the mechanisms underlying the connection between visfatin/Nampt and cancer. Further research is required in order to conclude whether visfatin/Nampt may be a therapeutic target in the pharmacological arsenal for cancer.

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Abbreviations

ADP	Mono-adenosine diphosphate
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMPK	5′ AMP-activated protein kinase
ATP	Adenosine triphosphate
BC	Breast cancer
BMI	Body mass index
Cdks	Cyclin-dependent kinases
C.I.	Confidence interval
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CRP	C-reactive protein
CtBPs	Mammalian COOH-terminal binding proteins
ER	Estrogen receptor
ERKs	Extracellular signal-regulated kinases
HL	Hodgkin lymphoma
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IGF	Insulin-like growth factor
IRS	Insulin receptor substrate
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
MM	Multiple myeloma
MPD	Myeloproliferative disorders
MMPs	Matrix metalloproteinases
NAD	Nicotinamide adenine dinucleotide
Nampt	Nicotinamide phosphoribosyltransferase
NHL	Non-Hodgkin lymphoma
NF-kB	Nuclear factor-kB
OR	Odds ratio
PARP	Poly (ADP-ribose) polymerase
PBEF	Pre-B-cell colony-enhancing factor
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
SirT	Silent mating type information regulation, sirtuin
SNPs	Single-nucleotide polymorphisms
STAT	Signal transducer and activator of transcription
TNF-α	Tumor necrosis factor-α

Tiam1	T-cell lymphoma invasion and metastasis-inducing protein 1
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor

Background

Cancer is one of the leading causes of deaths in the Western population. Beyond the established cancer predisposing factors such as heredity, radiation, tobacco smoking, infections, diet, diabetes mellitus, and other environmental parameters, high body weight is a well-known risk factor for several cancers, including breast, endometrial, esophageal, colon, hepatic, pancreatic, renal, prostate, and hematopoietic cancer [1–4].

Overweight and obesity (defined as a body mass index-BMI of ≥25 and ≥30 kg/m^2, respectively) have reached epidemic dimension with a prevalence of 60 % in many Western countries [3]. Novel studies have shown that the triad of overweight/obesity, metabolic syndrome, and adipocytokines such as leptin, adiponectin, and resistin is associated with cancer occurrence and prognosis [1, 5–10]. Indeed, population-based studies and meta-analyses have shown a 3 % increase in postmenopausal breast cancer risk per 1 kg/m^2 increase in BMI [11].

Potential biological factors associating overweight/obesity, specifically visceral obesity, with cancer could involve (1) obesity-induced chronic low-grade inflammation, (2) insulin sensitivity and the insulin-like growth factor system (IGF), (3) oxidative stress, (4) signaling transduction involving cancer cell promotion, (5) abnormal production of adipokines, and (6) synthesis and availability of endogenous sex hormones [1, 5]. Beyond its triglyceride-storing fat depot, adipose tissue is now seen as a real endocrine organ synthesizing several bioactive adipokines, a group of polypeptides regulating a wide range of physiological and pathological processes, such as appetite, insulin sensitivity, inflammation, reproduction innate and adaptive immunity, hematopoietic function, and vascular homeostasis [12–14]. Among adipokines, only adiponectin, leptin, and resistin have been extensively studied in cancer [1, 5–7, 15, 16]. In particular, hypoadiponectinemia, hyperleptinemia, and hyperresistenemia have been linked to cancer occurrence; however, the exact mechanisms remain obscure [1, 5–7]. In this book chapter, we will particularly focus on both intracellular and extracellular visfatin/Nampt (nicotinamide phosphoribosyltransferase) contribution to cancer pathophysiology and on the mechanisms underlying the connection between visfatin/Nampt and cancer.

Understanding Visfatin/Nampt Function

Visfatin/Nampt, also known as pre-B-cell colony-enhancing factor (PBEF), may be found both extracellularly in the plasma and intracellularly in the mitochondria, cytoplasm, and nucleus in the majority of cells [17, 18]. Based on the HUGO Gene

Nomenclature Committee, the official name of the gene and protein is Nampt [19]. In this book chapter, we will use the term visfatin/Nampt.

Visfatin/Nampt exerts a significant role in a wide range of metabolic functions, including energy metabolism in the cell, particularly nicotinamide adenine dinucleotide (NAD) production [20–22]. NAD could be synthesized either de novo from nicotinic acid, tryptophan and quinolinic acid or by the salvage pathway, whereas Nampt represents the rate-limiting enzyme in NAD production [23, 24]. The salvage pathway is more productive and faster and represents the basic pathway of NAD biosynthesis in mammals [23]. Hence, intracellular visfatin/Nampt plays a pivotal role in the enzymatic activity of a range of NAD-dependent enzymes involving several essential functions in cell survival and inflammation such as tumor necrosis factor-α (TNF-α) production [25]. Extracellular visfatin/Nampt acts as a multifaceted molecule: an adipocytokine, a growth factor, and an enzyme [26, 27].

The NAMPT gene in humans is located at 7q22, spans a length of 36.908 bp, is formed of 11 exons and 10 introns, and is translated into a 491 amino acid protein 52–57 kDa [18, 28, 29]. Extracellular Nampt has a slightly elevated molecular weight than intracellular Nampt [23]. The transcription process produces 19 different mRNAs, 14 alternatively spliced variants, and five unspliced forms. Three are the main mRNA transcripts comprising 2.0, 2.4, and 4.0 kb transcripts. NAMPT gene possesses a pseudogene on chromosome 10 [24, 27]. Till today, more than 730 single-nucleotide polymorphisms (SNPs) are observed in the human NAMPT gene with unclear biological consequences for the majority of them [22, 27, 30]. Intracellular visfatin/Nampt is autophosphorylated at histidine 247, a significant posttranslational modification, which augments its catalytic activity to bind nicotinamide [31]. Both intracellular and extracellular visfatin/Nampt undergo several posttranslational modifications, comprising ubiquitination and acetylation, with unclear functional importance [23]. NAMPT gene is highly conserved as an important sequence homology observed amid prokaryotic organisms such as *Haemophilus ducreyi* (30 %), primitive metazoans, and mammals such as mouse (95 %) and humans [13, 27, 32, 33].

Intracellular visfatin/Nampt is produced in the human heart, placenta, brain, lungs, liver, skeletal muscle, kidney, and pancreas with an important amount in muscle tissue [34]. Because extracellular visfatin/Nampt does not present a caspase I cleavage site nor a signal sequence related to the classical secretory pathway, it has been considered that extracellular visfatin/Nampt is released in plasma by cell death or via a nonclassical pathway by several cell types [20, 35–41].

Visfatin/Nampt gene and protein dysregulation have been encountered in the etiopathogenesis of a number of human disease entities. Visfatin/Nampt has been associated with obesity, diabetes mellitus, aging, neurodegeneration, atherosclerosis, sepsis, acute lung injury, rheumatoid arthritis, and cancer [22, 27, 30].

Adipose tissue, particularly visceral fat compared to subcutaneous one, constitutes one of the major sources of extracellular visfatin/Nampt [37, 42, 43]. In obese individuals, macrophages of visceral fat represent an important source of visfatin/Nampt production [44]. Recent data have shown that plasma visfatin/Nampt is elevated in obesity [45], particularly in obese women, children, and teenagers and in obesity-associated disorders such as metabolic syndrome, type 2 diabetes mellitus, nonalcoholic fatty

liver disease, chronic renal disease, coronary heart disease, polycystic ovary syndrome, and preeclampsia [20, 21, 46–49]. So far, results have been conflicting concerning correlations of plasma visfatin/Nampt with metabolic and anthropometric variables implying a potential role of visfatin/Nampt in the chronic low-grade inflammation observed in obesity and type 2 diabetes mellitus as a pro-inflammatory cytokine [20–22, 25, 26]. Both circulating visfatin/Nampt and adipocyte- and macrophage-derived visfatin/Nampt may modulate pathways implicated in the development of obesity and associated disorders by impacting on glucose and lipid metabolism, insulin sensitivity, chronic subclinical inflammation, and oxidative stress response.

Investigating the Evidence Associating Visfatin/Nampt with Cancer

Several epidemiologic studies depicted in Table 6.1 have revealed that hypervisfatinemia (enhanced serum visfatin/Nampt levels) in vivo is linked not only to the risk of obesity-associated malignancies, such as breast cancer (BC) [50–53], endometrial cancer [54–57], colorectal cancer [58–63], and prostate cancer [64], but also to the risk of male oral squamous cell [65], gastric [66, 67], and bladder cancer [68], astrocytoma/glioblastoma [69], and hematopoietic cancer [70, 71]. Our group has shown that circulating visfatin/Nampt was statistically significantly higher in postmenopausal women suffering from BC than in control participants and patients with breast benign lesions independently from BC established risk factors, anthropometric and metabolic parameters, as well as plasma concentrations of leptin and adiponectin. Further stratification by BMI has shown that the relationship between visfatin/Nampt and postmenopausal BC occurrence was evident among postmenopausal women with higher BMI after taking into account all predisposing factors [50, 51]. Serum visfatin/Nampt was a surrogate clinical marker of malignant potential and stage progression [27]. Serum visfatin/Nampt may be employed as potential diagnostic and prognostic marker in the arsenal of BC management. Moreover, we have shown that circulating visfatin/Nampt could offer additional data together with known tumor markers such as carcinoembryonic antigen and CA 15-3, specifically in discerning early-stage cases and estrogen-/progesterone-negative status in postmenopausal BC [51]. Hormone receptor status, lymph node infiltration, and advanced BC stage were the most important parameters characterizing circulating visfatin/Nampt concentration [51].

Nevertheless, not all studies have shown enhanced serum visfatin/Nampt levels in cancer patients in comparison to control participants [72–77]. These different results could be due to the study design (retrospective versus prospective), different laboratory methodologies detecting visfatin/Nampt, sample handling, different populations and ethnic groups, different types of cancer, different sample sizes (small sample size may lead to underpowered studies unable to detect true associations), and the adjustment or not for many confounders in the statistical analyses [78].

Table 6.1 Reported associations between circulating visfatin/Nampt with different types of cancer in recent comparative epidemiologic studies

Authors of study [references]	Type of study; cases/controls; population	Odds ratios (95% confidence intervals) or *p*-value	Additional data
Hematologic malignancies			
Multiple myeloma (*MM*)			
Dalamaga et al. [72]	Retrospective case control; 73/73; Greek population	0.89 (0.85–0.94) Adjusted for age, gender, BMI, and other adipokines	In MM patients, serum visfatin/Nampt was significantly lower compared to controls and was positively correlated with CRP, LDH, BMG, ESR, and disease stage. Visfatin/Nampt was significantly different among multiple myeloma stages with higher stages presenting higher visfatin/Nampt levels ($p<0.001$). No significantly different visfatin/Nampt levels were found among different paraprotein classes ($p=0.65$)
Cutaneous T-cell lymphoma (*CTCL*)			
Suga et al. [70]	Retrospective case control; patients with CTCL or atopic dermatitis (AD)/controls; Japanese population	$p<0.05$	Serum visfatin/Nampt concentrations in patients with AD or advanced stage CTCL were significantly enhanced compared to healthy controls. In CTCL patients, serum visfatin/Nampt concentrations were significantly reduced after treatment. Visfatin/Nampt expression in the adipose tissue in lesional skin of AD and advanced stage CTCL was enhanced compared to that of healthy controls
Childhood acute leukemia			
Skoczen et al. [73]	Prospective case control; 22/24, measurement before (22/22) and after (12/22) treatment with hematopoietic stem cell transplantation (HSCT) and before and after oral glucose tolerance test (OGTT); Polish children	$p<0.05$	Significantly lower median values of visfatin/Nampt concentrations at all time points in the OGTT were found in pre-HSCT children compared with control subjects. The median visfatin/Nampt level was significantly elevated after HSCT compared with that before HSCT. A decrease of visfatin/Nampt in leukemic children in complete remission has been found
Chronic lymphocytic leukemia (*CLL*)			
Audrito et al. [71]	Retrospective case control; 130/20 healthy donors; Italian population	$P<0.001$	Both intracellular and extracellular forms of visfatin/Nampt are increased in cells and plasma of CLL patients. Mean plasma visfatin/Nampt was significantly elevated in CLL patients than in healthy blood donors of comparable age Plasma visfatin/Nampt was positively correlated with Nampt mRNA expression in purified CLL cells, absolute lymphocytes, and circulating monocytes
Other malignancies			
Astrocytoma/glioblastoma			
Reddy et al. [69]	Retrospective case control; 80/9, 51 cases with glioblastoma; Indian population	$p<0.05$	Serum visfatin/Nampt levels of astrocytoma and glioblastoma patients were higher compared to controls. Moreover, in patients with astrocytoma, elevated levels of visfatin/Nampt correlated with tumor grade Visfatin/Nampt expression in the tumor tissue along with p53 co-expression was associated with poor survival

Bladder cancer (*BLC*)			
Zhang et al. [68]	Retrospective case control; 131 with transitional cell BLC/109; Chinese population	$P<0.001$ 2.85 (1.01–8.06), $p=0.048$ Adjusted for age, sex, smoking status, tumor stage, and tumor grade	Serum visfatin/Nampt level in patients with BLC was significantly higher than in the control group. Serum visfatin/Nampt was an independent prognostic factor for non-muscle-invasive BLC, with higher level (>14.74 ng/mL) indicating shorter recurrence-free survival rate
Breast cancer (*BC*)			
Dalamaga et al. [50]	Retrospective case control; 102/102; Postmenopausal Greek women	$p<0.001$ 7.93 (2.52–24.9), $p<0.001$ highest versus lowest quartile of visfatin/Nampt adjusting for age, date of diagnosis, education, BMI, waist circumference (WC), years with menstruation, parity/age at first full-term pregnancy, breast-feeding, family history of cancer, use of exogenous hormones, alcohol consumption, smoking status, homeostasis model assessment score, and serum leptin and adiponectin concentrations	Mean serum visfatin/Nampt levels were significantly higher in cases than controls. Elevated serum visfatin/Nampt levels were associated with PBC risk independently from BC known risk factors, anthropometric and metabolic parameters, as well as serum concentrations of other adipokines, particularly among overweight/obese individuals
Dalamaga et al. [51]	Retrospective case control; 103/103 and 51 patients with benign, breast lesions; Postmenopausal Greek women	$p<0.001$	Mean serum visfatin/Nampt was significantly higher in cases than in controls and patients with benign breast lesions. In cases, visfatin/Nampt was significantly associated with CA 15-3 ($p=0.03$), hormone receptor status ($p<0.001$), and lymph node invasion ($p=0.06$) but not with metabolic and anthropometric variables ($p>0.05$). In the multivariable regression analysis, the most significant predictors/determinants of serum visfatin/Nampt levels were the hormone receptor status, BC stage, and lymph node involvement Serum visfatin/Nampt outperformed CA 15-3 in discriminating between PBC cases with early cancer stage than those with late stage and in differentiating particularly patients with estrogen- and progesterone-negative breast tumors
Li et al. [52]	Retrospective case control; 248/100; Chinese women	$p<0.05$	Preoperative serum visfatin/Nampt level was substantially elevated in patients than controls. Elevated preoperative visfatinemia was an independent predictor of mortality, unfavorable outcome, disease-free survival, and overall survival. Visfatinemia presented high predictive value for mortality and unfavorable outcome

(continued)

Table 6.1 (continued)

Authors of study [references]	Type of study; cases/controls; population	Odds ratios (95% confidence intervals) or *p*-value	Additional data
Assiri et al. [53]	Retrospective case control; 82/68; Pre- and postmenopausal women; Saudi Arabian women	1.089 (1.062–1.116) $p=0.001$	There were significantly elevated concentrations of visfatin/Nampt in PBC patients than in age- and BMI-matched controls. Serum visfatin/Nampt showed positive significant correlation with BMI, WC, triglycerides, and glucose. Only in postmenopausal women, serum visfatin/Nampt was positively correlated with stage, tumor size, lymph node metastasis, and histological grading. In postmenopausal women, visfatin/Nampt was a risk factor for BC
Colorectal cancer (*CRC*)			
Nakajima et al. [58]	Retrospective case control; 115/115; Japanese population	2.985 (1.862–4.787) $p<0.01$	Serum visfatin/Nampt levels in cancer patients were significantly higher compared with age-, sex-, and BMI-matched controls. Visfatin/Nampt levels gradually increased with tumor stage progression, presenting a statistically significant correlation. Visfatin/Nampt may be a good biomarker of colorectal malignant potential and stage progression
Kosova et al. [74]	Retrospective case control; 20/20; Turkish population	$p>0.05$	No significant difference in visfatin/Nampt concentrations between cases and control participants. Serum visfatin/Nampt levels after treatment did not change significantly in both groups
Al-Harithy [59]	Retrospective case control; 90/98; Saudi Arabian women	$p<0.001$	Patients with colon cancer had significantly higher circulating visfatin/Nampt levels than controls. No correlation between visfatin/Nampt and tumor stage ($p=0.95$). Nonsignificant associations with anthropometric and metabolic parameters were observed for visfatin/Nampt. Visfatin/Nampt presented a significant correlation with HDL ($p=0.054$)
Chen et al. [60]	Retrospective case control; 358/286; Chinese population	3.37 (1.93–8.37) $p=0.011$ Early CRC, adjusted for potential confounding factors 2.38 (1.82–8.35) $p=0.015$ Advanced CRC, adjusted for potential confounding factors	Plasma visfatin/Nampt levels in patients with advanced and early CRC were higher compared to controls. Visfatin/Nampt correlated significantly with waist-to-hip ratio ($p<0.05$l) among cases and controls
Fazeli et al. [61]	Retrospective case control; 39/30; Iranian population	$p<0.001$	Patients with CRC presented significantly higher circulating visfatin/Nampt levels compared to age- and sex-matched controls, before and after adjustment for age, BMI, and waist-to-hip ratio
Tulubas et al. [62]	Retrospective case control; 30 patients with CRC/30 controls, 30 patients with adenoma; Turkish population	$p<0.001$	Serum visfatin/Nampt levels in patients with CRC were higher compared to controls and patients with adenoma. Serum visfatin/Nampt presented a positive correlation with serum resistin levels

Neubauer et al. [63]	Retrospective case control; 51/54 patients with polyps, nonactive inflammatory bowel disease, or irritable bowel syndrome served as controls; Polish population	$p=0.019$	Circulating visfatin/Nampt was significantly higher in patients with CRC than controls. Serum visfatin/Nampt was associated with lymph node metastasis, inflammatory, and angiogenic indices. There was no direct correlation between circulating visfatin/Nampt and its mRNA expression or protein concentration, either in tumor or in normal tissue Tumor visfatin upregulation was associated with metastasis, anemia, tumor location, inflammatory, and angiogenic indices
Endometrial cancer (*EC*)			
Tian et al. [54]	Retrospective case control; 234; Chinese population	$p<0.05$	Serum visfatin/Nampt was significantly elevated in EC patients than in healthy controls. Visfatin/Nampt expression was significantly higher in EC tissue than in normal endometrial tissue ($p=0.001$). Moreover, serum visfatin/Nampt levels were significantly positively correlated with tissue expression of visfatin/Nampt in EC patients. Elevated visfatin/Nampt expression correlated with myometrial invasion, advanced stage, and shorter survival
Luhn et al. [55]	Prospective nested case control; 167/327; PLCO Cancer Screening Trial, $N=78$, 216 participants; US American population	$p=0.046$ $p>0.05$ Adjusting for known endometrial cancer risk factors, including BMI and circulating estradiol levels	Median levels of visfatin/Nampt were higher in cases compared to controls Nonsignificant association between pre-diagnostic serum levels of visfatin/Nampt with postmenopausal EC risk was observed in multivariate analysis. However, there was an increased but not statistically significant EC risk in women belonging to the highest visfatin/Nampt tertile compared to the lowest one
Ilhan et al. [56]	Retrospective case control; 42/42; Turkish population	$p=0.011$ 1.091 (1.021–1.166) $p=0.010$	EC patients presented significantly higher visfatin/Nampt levels than controls In multivariate analysis, serum visfatin/Nampt elevation was associated with risk of myometrial invasion
Nergiz Avcioglu et al. [57]	Retrospective case control; 46/44; Turkish population	$p<0.001$	Serum visfatin/Nampt levels were higher in patients with EC compared to controls. In patients, visfatin/Nampt concentrations were positively correlated with age ($p=0.002$), BMI ($p=0.001$), fasting insulin ($p=0.002$), total cholesterol ($p=0.006$), triglyceride ($p<0.001$) levels, and HOMA-IR ($p=0.007$)
Esophageal cancer (*squamous cell carcinoma-ESCC*)			
Nakajima et al. [75]	Retrospective case control; 117/117; Japanese population	$p>0.05$	Similar visfatin/Nampt levels were observed in both cases and controls
Oral squamous cell carcinoma (*OSCC*)			
Yu-Duan et al. [65]	Retrospective case control; 51/57; Chinese male population	$p=0.002$ 1.367 (1.030–1.814), $p=0.03$ After controlling for anthropometric variables, blood pressure, lipid profile, betel quid chewing, drinking, and smoking status	Plasma visfatin/Nampt levels were elevated in patients with ESCC compared to age- and BMI-matched controls Visfatin/Nampt was independently associated with ESCC adjusting for known biomarkers. Visfatin/Nampt was positively associated with white blood cell and neutrophil count and was negatively correlated with hematocrit level. Furthermore, visfatin/Nampt was gradually increased with stage progression

(continued)

Table 6.1 (continued)

Authors of study [references]	Type of study; cases/controls; population	Odds ratios (95% confidence intervals) or *p*-value	Additional data
Gastric cancer (*GC*)			
Nakajima et al. [66]	Retrospective case control; 156/156; Japanese population	$p=0.0013$	Serum visfatin/Nampt levels were significantly higher in patients than in age- and gender-matched controls. No correlation between visfatin/Nampt and BMI was found in either patients or controls. Serum visfatin/Nampt levels gradually increased with stage progression ($p<0.001$)
Lu et al. [67]	Retrospective case control; 262/262; Chinese population	$p<0.05$	Preoperative plasma visfatin/Nampt concentrations were substantially higher in patients than in controls and correlated with invasion depth, lymph node metastasis, distant metastasis, peritoneal dissemination, tumor size, and tumor node metastasis stage. Elevated plasma visfatin/Nampt was an independent predictor for death and overall survival
Hepatocellular carcinoma (*HCC*)			
Chen et al. [76]	Prospective nested case control; 187/374; HBV carriers; Taiwanese population	$p>0.05$ After adjustment for metabolic factors and HBV-related factors	Serum visfatin/Nampt levels were not associated with an increased risk of HCC
Pancreatic cancer (*PaC*)			
Gasiorowska et al. [77]	Retrospective case control; 45/13; Polish population	$p>0.05$	No significant difference in serum visfatin/Nampt concentrations were found between cases and controls and among patients with and without diabetes mellitus. No significant correlations were observed between serum visfatin/Nampt and age, gender, BMI, smoking status, tumor localization, distant metastases, and pain
Prostate cancer (*PC*)			
Gomaa et al. [64]	Retrospective case control; 38/15 and 36 with benign prostatic hyperplasia(BPH); Egyptian population	$p<0.001$	PC patients presented significantly elevated levels of visfatin/Nampt compared to controls and patients with BPH In patients with PC, visfatin/Nampt presented a significant positive correlation with Gleason score ($p=0.019$). Visfatin/Nampt, BMI, and PSA were the major significant determinants of Gleason score

BMG B-2 microglobulin, *BMI* body mass index, *CRP* C-reactive protein, *ER* estrogen receptor, *ESR* erythrocyte sedimentation rate, *HBV* hepatitis B virus, *HDL* high-density lipoprotein, *HOMA-IR* homeostasis model assessment index for insulin resistance, *LDH* lactate dehydrogenase, *Nampt* nicotinamide phosphoribosyltransferase, *PBC* postmenopausal breast cancer, *PLCO* Prostate, Lung, Colorectal, Ovarian Cancer Screening Trial, *PSA* prostate-specific antigen

The relation of visfatin/Nampt with cancer is also in accordance with histopathological studies depicting associations between malignant tissue intracellular visfatin/Nampt expression and cancer stage and prognosis [27]. Indeed, enhanced intracellular visfatin/Nampt expression was shown in breast, colorectal, ovarian, gastric, colorectal, prostate, well-differentiated thyroid, and endometrial cancer as well as in brain tumors and lymphomas [69, 79–83]. Specifically, intracellular visfatin/Nampt expression increased cellular stromal cell-derived factor-1 levels in malignant colon cells enhancing cancer progression [84]. Intracellular visfatin/Nampt expression was 13 times elevated in gastric malignant tissue than in benign gastric tissue [85].

Higher intracellular visfatin/Nampt expression is correlated with myometrial invasion, late stage, and shorter survival in women with endometrial carcinoma [54]. Augmented intracellular visfatin/Nampt was related with increased astrocytoma grade and tumor stage in hepatocellular, gastric, colorectal, and male oral squamous cell carcinomas [58, 65, 66, 69, 86].

In summary, the elevated expression of intracellular visfatin/Nampt in cancer tissues in conjunction with higher serum visfatin/Nampt concentration in advanced cancer stage and grade highlights the contribution of visfatin/Nampt not only to cancer promotion but also to cancer progression and prognosis. Hence, visfatin/Nampt could be a candidate clinical marker for cancer development and progression.

Uncovering the Role of Visfatin/Nampt in Cancer Pathophysiology

Visfatin/Nampt could exhibit neoplastic actions by playing the following roles: (1) visfatin/Nampt is a pro-inflammatory adipocytokine connecting obesity with cancer; (2) visfatin/Nampt is a metabolic mediator connecting insulin sensitivity with cancer; (3) visfatin/Nampt, particularly intracellular visfatin/Nampt, is an enzyme in energy metabolism, circadian clock, and cell longevity through intracellular NAD production; (4) visfatin/Nampt is an antiapoptotic and proliferative factor; (5) visfatin/Nampt is a pro-angiogenic and metastatic factor; and (6) visfatin/Nampt may be a hormonal mediator to certain types of cancer. Figure 6.1 depicts the visfatin/Nampt-mediated NAD biosynthesis as well as visfatin/Nampt's neoplastic actions.

Visfatin/Nampt Is a Pro-inflammatory Adipocytokine Connecting Obesity with Cancer

Although visfatin/Nampt is enhanced in overweight/obese individuals, no association is found between serum visfatin/Nampt and anthropometric (BMI, waist circumference, waist-to-hip ratio) or metabolic parameters in several epidemiologic studies [20, 21, 87, 88]. In this context, we could hypothesize

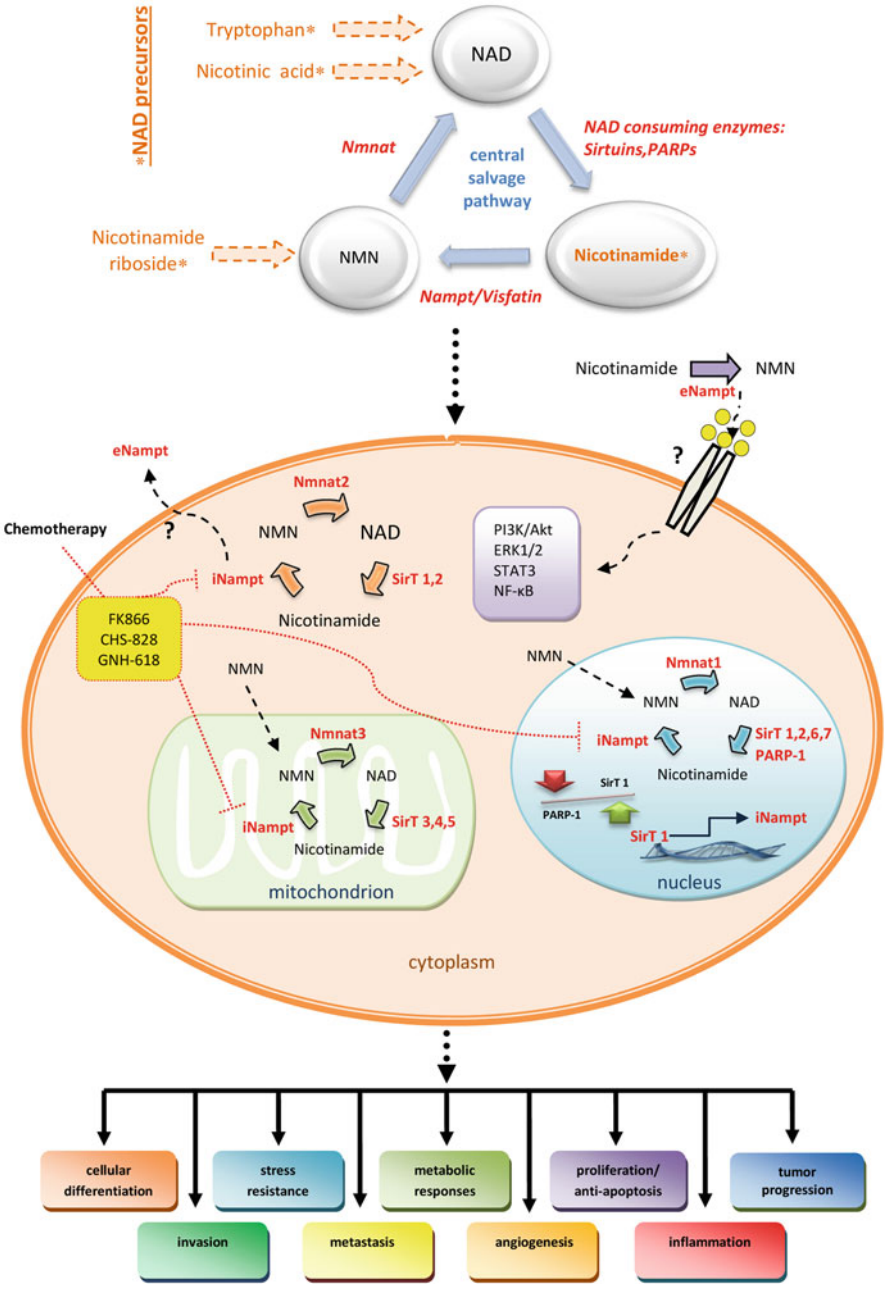

Fig. 6.1 Nampt-mediated NAD biosynthesis and putative model of Nampt role in cancer cells. Nampt can be both extracellular in plasma and intracellular in different cellular sections, playing a pivotal role in NAD biosynthesis. NAD may be formed de novo from precursors such as nicotinic acid, tryptophan, and nicotinamide riboside or by the salvage pathway where Nampt catalyzes the rate-limiting step of NAD biosynthesis. Its intracellular function focuses on the regulation of the activity of NAD-consuming enzymes such as sirtuins and PARPs, thus affecting inflammation, stress resistance, DNA repair, and metabolic responses. eNampt exerts its action through binding to an unknown receptor that regulates a variety of molecular signaling pathways, such as PI3K/Akt, ERK1/ERK2, STAT3, and NF-kB, thereby exhibiting proliferative, antiapoptotic, pro-inflammatory,

another potential association between serum visfatin/Nampt and vascular inflammation induced by obesity and insulin resistance. Indeed, it has been reported that visfatin/Nampt represents a very potent IL-6 inducer both in vitro and in vivo [21, 89]. IL-6 is implicated in the insulin resistance pathogenesis associated with central obesity [20]. Visfatin/Nampt could help the perpetuation of cell-mediated inflammation at the location of enhanced visfatin/Nampt concentrations. Experimental evidence has shown that visfatin/Nampt induces macrophage survival via the IL-6 release as well as inhibits neutrophil apoptosis in sepsis [17, 90].

Visfatin/Nampt promotes the synthesis and release of a variety of pro-inflammatory cytokines in human mononuclear cells upregulating the production of IL-1α, IL-6, and TNF-α [21, 89]. Plasma visfatin/Nampt levels are upregulated in many inflammatory disorders and are associated with inflammation degree [20]. Furthermore, exogenous visfatin/Nampt activates leukocyte adhesion to endothelial cells by enhancing the expression of cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin, which are key players in leukocyte recruitment [22, 91]. The main inflammatory pathways induced by visfatin/Nampt are the pro-inflammatory transcription factor nuclear factor-kB (NF-kB), the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinases (ERK) 1/2 and p38 as well as phosphatidylinositol 3-kinase (PI3K), and the intracellular generation of reactive oxygen species [48, 91]. Based on the fact that upregulated activities of NF-kB have been involved in cancer pathogenesis, all the above data may provide evidence for a probable visfatin/Nampt implication in the pathogenesis of vascular inflammation connecting obesity – a state of chronic low-grade subclinical inflammation – to malignancies.

Visfatin/Nampt Is a Metabolic Mediator Connecting Insulin Sensitivity with Cancer

Visfatin/Nampt was initially introduced as an insulin-mimetic molecule promoting insulin signaling by binding insulin receptor at a different location than insulin. However, data reproducibility regarding visfatin/Nampt beneficial effects on activating the insulin receptor was questioned, and the original paper by Fukuhara et al. was retracted from the journal *Science* [92]. Later, visfatin/Nampt glucose-lowering

Fig. 6.1 (continued) pro-angiogenic, and metastatic properties. Interestingly, Nampt inhibitors such as FK866, CHS-828, and GNE-618 have shown efficacy in various models of cancer. *Akt* v-Akt murine thymoma viral oncogene homolog, *eNampt* extracellular Nampt, *ERK1* extracellular signal-regulated kinases, *iNampt* intracellular Nampt, *NAD* nicotinamide adenine dinucleotide, *Nampt* nicotinamide phosphoribosyltransferase, *NF-kB* nuclear factor-kB, *Nmnat* nicotinamide mononucleotide adenylyltransferase, *NMN* nicotinamide mononucleotide, *PARP* poly (ADP-ribose) polymerase, *PI3K* phosphatidylinositol 3-kinase, *SirT* silent mating type information regulation, sirtuin, *STAT* signal transducer and activator of transcription

properties were reported in human osteoblasts, whereas visfatin/Nampt exerted tyrosine phosphorylation of the insulin receptor, insulin receptor substrate (IRS)-1, and IRS-2 in a close resemblance to insulin action [93].

Extracellular visfatin/Nampt could not present direct hypoglycemic actions in vitro and in vivo but an important NAD biosynthetic activity. Indeed, both intracellular and extracellular visfatin/Nampt-mediated NAD synthesis may play a significant role in the regulation of glucose homeostasis and normal β pancreatic cell secretion. Visfatin/Nampt is crucial for the modulation of glucose-stimulated insulin release [94]. Revollo et al. have reported that visfatin/Nampt haplodeficient mice exhibit reduced plasma visfatin/Nampt concentrations and defects in glucose-stimulated insulin production by the pancreatic β cells [94]. More scientific evidence is required to highlight the controversial role of extracellular visfatin/Nampt insulin-mimetic function. Hyperinsulinemia and insulin resistance in the frame of overweight/obesity have been associated with cancer occurrence [95]. For example, upregulated serum visfatin/Nampt concentrations reported in postmenopausal BC patients could be due to the function of visfatin/Nampt as a metabolic mediator improving or adjusting for hyperinsulinemia and insulin resistance in these cases [8, 26, 50, 51].

Visfatin/Nampt, Particularly Intracellular Visfatin/Nampt, Is an Important Enzyme in Cellular Energy Metabolism

Due to its pivotal role in the recycling pathway allowing NAD synthesis from nicotinamide, intracellular visfatin/Nampt takes up a central position in controlling the function of many NAD-dependent enzymes [25]. NAD, a universal energy- and signal-carrying molecule [22], and its phosphorylated form, NADP, are mandatory in many intracellular mechanisms like DNA reconstruction, redox reactions, transcriptional regulation, G-protein-coupled receptor signaling, intracellular calcium-assembling molecules, chromatin dynamics regulation, circadian rhythms, telomerase function, mono-adenosine diphosphate (ADP)-ribosylation in immune activity, and function of poly-ADP ribosyltransferases (PARPs) and deacetylases (sirtuins) which are implicated in cell longevity, genomic stability, and cytokine functions [24, 26].

Under the impact of intracellular visfatin/Nampt, sufficient concentrations of NAD regulate silent mating type information regulation (SirT or sirtuin)-6 function, which consecutively controls positively TNF-α mRNA translation facilitating cell survival [25, 96]. Indeed, TNF-α may promote both cell death and longevity signals [96]. Intracellular visfatin/Nampt function augments cellular proliferation, places the balance toward cellular longevity after genotoxic stress, and modulates the circadian clock engine of significant key transcription factors [21]. SirT1, which is the best characterized molecule of the sirtuin family, is a NAD-dependent histone deacetylase, occupies a significant function as a survival-promoting protein, and is overexpressed together with intracellular visfatin/Nampt in breast, gastric, colon, liver, prostate, and pancreatic malignancies [24, 26, 97]. Intracellular visfatin/Nampt upregulation augments intracellular NAD, upregulates SirT1-mediated p53

deterioration, and generates resistance to hydrogen peroxide-induced cell destruction in human vascular smooth muscle cells [98]. Furthermore, SirT1 stimulates oncogenesis via dissipating p53, retinoblastoma protein, and phosphatase and tensin homolog protein (PTEN) functions and promotes epithelial-to-mesenchymal transition by enhancing cell migration [97, 99–103].

The interplay between intracellular visfatin/Nampt and PARP-1, which exhibits divergent cellular functions such as DNA repair regulation, transcription, intracellular signaling, protein degradation, proliferation, and differentiation, plays an important function in cell longevity [104, 105]. Consequently, intracellular visfatin/Nampt and PARP-1 upregulation is often shown in the same cancer [24, 106, 107]. Intracellular visfatin/Nampt neutralization by the specific Nampt inhibitor APO866 has been shown to downregulate PARP-1 function leading to reduced myeloma cell growth and osteoclast activity [108]. Intracellular visfatin/Nampt could contribute to cell survival by reducing PARP-1 activity through upregulation of SirT1 function, which in turn negatively regulates PARP-1 at the transcriptional level [109]. PARP-1 and SirT1 could perform in an antagonistic manner competing for nuclear NAD [110, 111]. More studies are needed in order to unfold the exact molecular process of the interconnection between intracellular visfatin/Nampt, PARP-1, and SirT1.

Additional metabolomic data are required in order to elucidate NAD generation and bioenergetics including its time-spatial dynamics in the metabolism of cancer cells [112].

Visfatin/Nampt Is an Antiapoptotic and Proliferative Factor

Exogenous administration of visfatin/Nampt has been shown to enhance the proliferation and DNA generation rate of MCF-7 human BC cells [113]. Also, exogenous visfatin/Nampt could trigger cell and tumor growth in MDA-MB-231 BC cell line and in nontransformed MCF10A mammary epithelium by upregulating Notch1, thus highlighting that the Nampt-Notch1 axis could contribute to cancer growth via the activation of the NF-kB [114]. Although its various properties in NAD biosynthesis, extracellular visfatin/Nampt may influence oncogenesis by upregulating the cell proliferation rate through enhancement of cell cycle progression and by increasing the expression of genes that contribute to angiogenesis and metastasis. Exogenous visfatin/Nampt stimulated mRNA levels of cyclin D1 and cyclin-dependent kinase (cdk)2, which are well-known modulators of the G1-S progression [113]. Extracellular visfatin/Nampt also upregulated an antiapoptotic interleukin-6/signal transducer and activator of transcription (STAT)3-mediated cell survival in macrophages through a nonenzymatic mechanism that could account for macrophage physiologic alterations mediating obesity actions in inflammation and oncogenesis [17]. Additionally, in prostate cancer, extracellular visfatin/Nampt upregulated PC3 cell proliferation by inducing the MAPKs ERK 1/2 and p38 signaling pathways [115]. To this end, it would be of importance to find the receptor of extracellular visfatin/Nampt and determine its signaling mechanism in tumor growth.

Visfatin/Nampt Is a Pro-angiogenic and Metastatic Factor

Increased serum visfatin/Nampt concentrations were seen in postmenopausal BC patients with advanced stage and worse prognosis [51]. Elevated intracellular visfatin/Nampt expression in BC tissues was linked to poor prognosis [80]. Also, extracellular visfatin/Nampt could be a surrogate clinical marker for stage progression in colorectal cancer [58]. Aberrant angiogenesis is a key process for tumor growth and expansion. Exogenous visfatin/Nampt promotes angiogenesis through stimulation of MAPK ERK-dependent pathway via endothelial fibroblast growth factor-2 and upregulates vascular endothelial growth factor (VEGF) through MAPK and PI3K/Akt signaling pathways [20, 116, 117]. Exogenous visfatin/Nampt also stimulates the synthesis of matrix metalloproteinases (MMPs) 2 and 9 and VEGF genes highlighting its functions in BC angiogenesis and metastasis [113]. MMPs 2 and 9 are involved in the destruction of the extracellular matrix, indicating a potential function of visfatin/Nampt in cell migration, invasion, and metastasis [115]. Recent data have shown that exogenous visfatin/Nampt stimulates prostate malignant cells' proliferation and synthesis of MMP genes linked to advanced cancer stage, higher malignant cell invasion, and metastatic formations [115].

Intracellular visfatin/Nampt may influence metastatic activity and cell adhesive roles in regulating αvβ3 and β1 integrins in BC [118]. Moreover, increased expression of the human NAMPT gene in BC cells is influenced by the hypoxia-inducible factor-1, a key mediator in tumor growth [119]. Hypoxia, which represents a typical consequence of tumor growth, serves to proliferate several molecular pathways including angiogenesis and glycolysis [119]. Hence, a hypoxic milieu within tumor mass may initiate visfatin/Nampt expression which, in turn, acts as a pro-angiogenic mediator in tumor growth and propagation. Enhanced intracellular visfatin/Nampt expression increases intracellular NAD concentration promoting the binding of mammalian COOH-terminal binding proteins (CtBPs), CtBP1 and CtBP2, to its cellular targets, thus influencing invasive behavior, epithelial-to-mesenchymal transition, cell migration through the T-cell lymphoma invasion and metastasis-inducing protein 1 (Tiam1), and apoptosis resistance in cancer cells with concomitant suppression of the anti-oncogene products (PTEN, E-cadherin, etc.) [120–123]. Thus, hypoxia in conjunction with intracellular visfatin/Nampt could function cooperatively leading to tumor progression via CtBP stimulation.

Visfatin/Nampt May Be a Hormonal Mediator to Certain Types of Cancer

Obese postmenopausal women exhibit an increased synthesis of estrogens. Obesity -related BC is often estrogen receptor (ER)-positive [124]. A recent study has shown that estrogens and progesterone used in combination synergistically enhance the expression of the NAMPT gene in 3T3-L1 cells [125]. Estrogens and/or progesterone

may upregulate intracellular visfatin/Nampt in adipocytes and pre-adipocytes of the breast gland. Excessive extracellular visfatin/Nampt release by adipocytes may function in an autocrine or paracrine manner and influence susceptible breast epithelium tumor growth and expansion [26]. Additional studies are required employing human adipocyte cell lines from overweight/obese and lean subjects in contrast to murine 3T3-L1 adipocytes that could not liberate the same levels of extracellular visfatin/Nampt as human adipocytes do. Interestingly, another study revealed that exogenous visfatin/Nampt did not alter ER-α synthesis implying that extracellular visfatin/Nampt stimulates BC cell growth via a distinct pathway from estrogen action [113]. Additional research is required to investigate the exact visfatin/Nampt signaling pathway in BC.

Developing Preventive and Therapeutic Strategies

Preventive Strategies

Elevated BMI and weight gain in adult life, which represent modifiable risk factors, are related to a higher cancer risk [1, 126, 127]. A small but important percentage of cancer cases in Western countries may be preventable through increasing physical activity levels; maintaining a healthy weight; following a diet with high intake of fruits, vegetables, and olive oil; and reducing alcohol intake [1, 9, 12]. Visfatin/Nampt, which is related to cancer occurrence, could be modifiable by weight loss, physical exercise, and diet [20, 21]. Particularly, weight control and aerobic exercise are related to a significant reduction in serum visfatin/Nampt concentrations and visfatin/Nampt liver expression [128]. Higher serum visfatin/Nampt levels in morbidly obese subjects are decreased after gastric banding [47]. Hence, although visfatin/Nampt is not only adipose-cell-derived, it is responsive to adiposity changes. Glycemic control may reduce not only glycosylated hemoglobin but also serum visfatin/Nampt levels [129]. Interestingly, cholesterol-lowering drugs may restore adipose tissue endocrine function [130]. Some nutraceuticals, such as curcumin, have also been reported to decrease mRNA and protein levels of visfatin/Nampt [131].

Regarding screening and monitoring, at present, there is no good serum tumor marker available for an early cancer detection. The most widely used serum clinical makers in the diagnosis, management, and surveillance of cancer patients are prostate-specific antigen, α-fetoprotein, and CA-12.5. Specificity and sensitivity issues preclude the use of serum tumor biomarkers for detecting in situ or early-stage invasive disease [132]. We have found that, despite the outperformance of CA 15-3 in discriminating women with postmenopausal BC from women with benign breast lesions and healthy controls, serum visfatin/Nampt was more sensitive in early-stage BC compared to CA 15-3 and may discriminate better tumors with negative hormone receptors [51]. Serum visfatin/Nampt may be a promising surrogate clinical marker in cancer reflecting advanced stage, adverse prognosis, and inflammatory state [24]. However, additional prospective and longitudinal studies are required in order to determine the prognostic value of visfatin/Nampt in the arsenal

of cancer monitoring and prognosis. Further investigations are required for the development of reliable laboratory techniques (enzyme-linked immunosorbent and electrochemiluminescence immunoassays, etc.) to determine the physiologic and pathophysiological relevance of serum visfatin/Nampt. Which visfatin/Nampt levels should be considered abnormal needs also to be determined as visfatin/Nampt concentrations may fluctuate with age, race, several pre-analytical parameters, and assay methodology [20–22, 133].

Similar to several new assays [134], international standardization of concentrations and assay procedures regarding visfatin/Nampt is also required in the future.

Therapeutic Strategies

Based on the association of visfatin/Nampt with cancer and the overexpression of intracellular visfatin/Nampt in several human cancers, novel therapeutic approaches may be implemented [135]. Targeting visfatin/Nampt inhibition, either by antibody neutralization, by antisense oligonucleotides, or by antagonism of the putative Nampt receptor, could be an effective therapeutic strategy in malignancies, particularly in depleting intracellular NAD and downregulating the tumor inflammatory microenvironment. The downregulation of NAMPT gene expression may be a novel anticancer strategy. Curcumin (diferuloylmethane), a polyphenol derived from the plant turmeric, which possesses anti-inflammatory and antioxidant properties, has been reported to downregulate NAMPT gene expression in human BC cells via, partly, a NF-kB-dependent pathway [131]. If visfatin/Nampt receptor and visfatin/Nampt-induced signaling pathways are clearly mapped out, inhibition of visfatin/Nampt downstream targets may be further evaluated in malignancy therapeutics.

Visfatin/Nampt biochemical inhibition causing cellular NAD depletion, especially in tumor cells that rely on nicotinamide to produce NAD, is effective in many cancer models including a mouse breast carcinoma model [20–22, 25]. Anti-visfatin/Nampt agents such as FK866 (or APO866), CHS-828, and GNE-618 suppressed cancer growth in a broad range of cancer cell lines by downregulating NAD levels, causing metabolic collapse and stimulating apoptosis [21, 136, 137]. In vitro and in vivo studies have shown that APO866 administration abrogated cancer growth in animal models of hematologic malignancies without important toxicity [138]. This highly specific and noncompetitive visfatin/Nampt inhibitor provoked cell death through apoptosis with a gradual NAD depletion in HepG2 human hepatic carcinoma cells [24, 139]. In vitro, the expression of the NADase CD38 was an important factor in the sensitivity of pancreatic cancer cells to the visfatin/Nampt inhibitor FK866 [140]. Intracellular visfatin/Nampt inhibition may also decrease adenosine triphosphate (ATP), SirT1, and PARP-1 activities in cancer than benign cells [141]. Recently, many visfatin/Nampt inhibitors are in phase I and II clinical trials showing promise [141–144]. Visfatin/Nampt inhibitors may be promising anticancer agents, but cancer cell resistance to these agents has not been studied in depth. Acquired resistance to visfatin/Nampt inhibitors has been associated with visfatin/Nampt mutations located in the vicinity of the active site or in the dimer interface of visfatin/Nampt [137, 145].

However, a new design of next-generation anti-visfatin/Nampt agents may ameliorate their therapeutic value by escaping resistance mechanisms [137].

Since most cancer cells exhibit extended PARP activation through DNA damage and show higher energy consumption, they may present vulnerability to NAD depletion [136]. Recent data have shown that the conjunction of a visfatin/Nampt inhibitor with another chemotherapy agent induces "synthetic lethality," whereas the neutralization of two proteins may cause cell death [146, 147]. Indeed, visfatin/Nampt and PARP-1 inhibitor (e.g., olaparib) combinations may be promising in triple-negative BC therapeutics [148].

While understanding the link of visfatin/Nampt to cancer may uncover novel therapeutic targets, lifestyle improvement remains the most significant anticancer component, particularly in obesity-related cancer. Despite the unknown consequences of prolonged serum visfatin/Nampt concentrations, physical activity, weight loss, and a Mediterranean-based diet with consumption of vegetables, fruits, and nuts related with lower visfatin/Nampt levels may reduce cancer occurrence.

Additional mechanistic and epidemiologic investigations are needed to explore if visfatin/Nampt levels are altered in cancer and whether visfatin/Nampt in conjunction with hormonal factors is at the intersection of overweight/obesity and cancer promotion. More research will be conducted to determine serum visfatin/Nampt clinical utility as a clinical surrogate biomarker and to investigate whether NAMPT genetic polymorphisms are clearly associated with cancer prevalence. Finally, mechanistic research will be implemented to explore the unknown epigenetic modulation of the NAMPT gene and to discover the visfatin/Nampt receptor and its critical signaling pathways in cancer promotion.

In conclusion, this book chapter provides evidence for a connection between visfatin/Nampt, obesity, and malignancies. Advances in visfatin/Nampt pathophysiology and visfatin/Nampt inhibitors may hold promise for visfatin/Nampt use as a potential cancer biomarker and therapeutic target. Simultaneously, many issues need to be elucidated in order to unmask the ontological role of visfatin/Nampt in cancer pathophysiology. Advances in the area of translational research could lead to important benefits to overweight/obese individuals who are at increased cancer risk.

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Chapter 7
Apelin and Cancer

Stefanie Kälin and Roland E. Kälin

Abstract Over the past decades, obesity became a tremendous socioecological problem, reaching epidemic dimensions and the number of accompanying comorbidities including type 2 diabetes and cardiovascular diseases can be extended by different types of cancer. The small peptide apelin is involved in key physiological processes such as angiogenesis, fluid homeostasis, and cardiovascular function. As a relatively new adipokine, apelin also exhibits metabolic functions in regulating insulin sensitivity and glucose metabolism. In rodents and humans, apelin serum levels seem to correlate with the nutritional status and even more interesting, apelin expression levels are increased in many different cancers due to its proangiogenic capacities, supporting a role of apelin as diagnostic biomarker in cancer and its potential use in anticancer therapies by blocking tumor neovascularization. This chapter highlights the role of apelin in both physiological and pathological conditions like tumorigenesis, especially in brain tumor development and reviews apelin signaling in metabolic disorders like obesity-related malignancies, to identify possible associations between the adipokine apelin and tumor progression.

Keywords Apelin • Apelin receptor • Angiogenesis • Cancer • Glioblastoma • Metabolism • Obesity • Glycolysis

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Identification of the Apelin Receptor and Its Endogenous Ligand Apelin

The human apelin receptor gene (*APLNR*; also known as APJ for putative receptor protein related to the angiotensin II receptor-like 1) was coincidently cloned with primers designed to obtain the vasopressin receptor or one of its subtypes in 1993 and found to encode a protein related to the angiotensin II receptor type 1 (AT1R) [1]. A *Xenopus* apelin receptor ortholog, called Msr (mesenchyme-associated serpentine receptor) was subsequently identified leading to the cloning of a cDNA with sequence homology to angiotensin receptors and CXC-chemokine receptors [2]. Msr expression is found in blood vessels of venous as well as arterial origin. Vascular expression is however transient as Msr expression is downregulated once endothelial cells differentiate in the cardiovascular system. Thus, Msr expression was associated with the endothelial cell lineage, indicating a role in vascular development and ever since used as a vascular marker in *Xenopus* embryology [3–5]. In the following years, mouse and rat apelin receptor genes were identified based on the *Xenopus* and human apelin receptor sequences [6–9].

Taking advantage of a novel screening method for ligands of orphan G-protein coupled receptors (GPCR), namely, testing various tissue extracts against stable cell lines expressing the designated orphan GPCR in an extracellular acidification assay, Tatemoto et al. identified apelin as bioactive peptide acting as ligand for the apelin receptor from bovine stomach extract [10]. Despite the structural relationship of the apelin receptor to angiotensin receptors, angiotensin II was found not to bind to the apelin receptor. In contrast, bioactive peptides derived from the apelin preproprotein (apelin-77) bound tightly to the apelin receptor, inducing acidification of the cytosol and internalization of the receptor [10, 11]. Apelin isoforms include apelin-36, apelin-17, apelin-13, and the pyroglutamylated (Pyr^1)apelin-13 [10]. Mature apelin-36 and apelin-13, consisting of the C-terminal region of apelin-77, were most active in the acidification assay with the binding affinity of apelin-36 being higher (IC_{50} of 5.4 nM) than the one of apelin-13 (IC_{50} of >100 nM) [8]. The presence of these two peptides in tissue extracts was confirmed by immunoreactivity to monoclonal antiapelin antibodies [12].

The diversity of apelin peptides may result from posttranslational maturation of a preproapelin, but not much is known about the cellular or extracellular events following signal peptide cleavage in the endoplasmic reticulum. At the catabolic level, a close homolog of angiotensin I-converting enzyme (ACE), named ACE2, cleaves apelin-36 and hydrolyses apelin-13 with high catalytic efficiency [13].

Interestingly, cloning of the *Xenopus* apelin gene and comparison of its sequence to the one of its vertebrate orthologs revealed that the mature apelin-13 peptide has remained invariant during 360 million years of tetrapod evolution, while the longer apelin-17 and apelin-36 peptides differ considerably [4]. The invariant amino acid residues shared by the longer forms are confined to the predicted proteolytic cleavage sites. Apelin-13 therefore may represent the primary physiologically important protein product while apelin-36 may function as precursor with some biological activity until undergoing further proteolysis to yield the fully active apelin-13 peptide [4, 14, 15].

Apelin and Apelin Receptor Expression and Signaling

Apelin Expression in Rodent and Human Adult Tissue

Northern blot and in situ hybridization analyses of adult rat tissues indicate that both apelin and its receptor are coexpressed in central and peripheral tissues, including brain, spinal cord, lung parenchyma, heart, kidney, liver, testis, ovary, mammary gland, and adipose tissues [8, 9, 12, 16]. Immunohistochemical experiments using polyclonal antiapelin antibodies on rat and mouse tissue revealed apelin peptides in adipose tissue, endothelia of small arteries of the mesenterium, omentum, heart, lung, gastrointestinal tract, spleen, pancreas, and kidney [15, 17, 18]. In the liver, positive staining was also seen in the endothelia of the portal and central veins and in Kupffer cells [17]. Moreover, preproapelin mRNA upregulation was detected at the leading edge of vessel formation in the mouse retina [19]. Within endothelial cells, apelin is present in the endoplasmic reticulum, Golgi complex, and secretory vesicles, suggesting synthesis of apelin via the constitutive pathway [15].

In humans, apelin gene expression was also reported in central and peripheral organs. Examples for the latter are placenta, with highest preproapelin expression, heart, lung, and kidney [18]. Corresponding to the murine expression profile of apelin, it could be immunohistochemically detected in endothelial cells, e.g., of the coronary artery and saphenous vein, as well as in blood vessels in heart, kidney, adrenal gland, and lung [15]. Northern blot analysis revealed high central preproapelin mRNA expression in thalamus, hypothalamus, frontal cortex, and midbrain [14]. Apelin was found in another human study throughout the central nervous system with highest expression in spinal cord, corpus callosum, amygdala, substantia nigra, and pituitary gland [18]. Similar mRNA expression profiles can be found in mouse and rat brain tissue [18]. (Pyr^{1})apelin-13 is the predominant isoform that is found in rat whole brain and hypothalamus, while apelin-17 is found in lower levels in the hypothalamus [20]. Apelin synthesizing neurons can be found in hypothalamic nuclei including the paraventricular and arcuate nuclei [11, 21], which harbor some of the key circuits responsible for nutrient sensing and metabolic control.

Apelin Receptor Expression

Mouse and rat apelin receptor genes were identified in the late 90s on the basis of the *Xenopus* and human apelin receptor sequences [6–9] and similar findings regarding receptor expression have been reported in the mouse embryo [6]. In situ hybridization of the mouse retina in postnatal stages confirmed that the apelin receptor is downregulated to a lower expression level once the retinal vessels become mature [19]. Expression of the apelin receptor protein in the vasculature could at first be indirectly revealed by the presence of apelin-binding sites in human heart and saphenous vein [22]. Expression of the apelin receptor by endothelial cells [2, 6] is associated with nitric oxide (NO) release

leading to vasodilation and peripheral hypotension [14, 17] and its prominent expression in the embryonic vasculature implicates important roles in the control of blood vessel development [6] (see section "Apelin Signaling in Cardiovascular Formation").

A rather unappreciated area of apelin receptor expression is the central nervous system. Initial study of O'Dowd and colleagues found apelin receptor transcripts in the human brain, which was confirmed and refined to certain areas of the brain by northern blot analysis [1, 23]. In situ hybridization analysis of adult rat brains discovered apelin receptor mRNA expression in the hypothalamic paraventricular and supraoptic nuclei as well as in the pituitary, in the pineal gland and other extrahypothalamic structures, suggesting an involvement of apelin receptor signaling in the regulation of hormone release, circadian rhythm, and water and food intake [7, 9].

Finally, immunocytochemical staining and quantitative polymerase chain reaction revealed abundant apelin receptor expression especially at high levels in cultured neurons and oligodendrocytes and at lower levels in astrocytes [24]. However, possible physiological functions of apelin signaling in the central nervous system are largely neglected with some exceptions reporting positive effects on neuroprotection (see section "Apelin-Mediated Signaling").

Apelin-Mediated Signaling

GPCRs are the largest family of cell-surface receptors in the genome and mediate many of the cell–cell communication processes in the human body. Thus, their dysfunction results in several known diseases [25] and the broad variety of extracellular activators or ligands—including ions, amino acids, hormones, and neurotransmitters—is one reason why GPCRs are most effectively exploited by the pharmaceutical industry, with approximately 60 % of all drugs acting on one or more GPCRs [26].

The identification of apelin via an acidification-based screening strategy suggested that apelin signaling suppresses forskolin-stimulated cyclic adenosine monophosphate (cAMP) production in apelin receptor-expressing Chinese hamster ovary (CHO) cells through an inhibitory G_i protein [10, 27]. This finding was confirmed by a cAMP enzyme immunoassay, showing that apelin-36, apelin-17, and apelin-13 were inhibiting cAMP synthesis through G_i, but not G_s or G_q proteins [16]. Moreover, the elevation of extracellular acidification was suppressed by pertussis toxin, a specific inhibitor for G_i, or by methyl-isobutyl amirolide, a specific inhibitor of the Na^+/H^+ exchanger [8]. Apelin receptor coupling to G_i explains various intracellular effects following apelin binding to its receptor, such as adenylyl cyclase inhibition [16, 28], increase of intracellular calcium concentrations [24], and activation of extracellular-regulated kinases (ERKs) [27], which are critically involved in cell division and which are often deregulated during oncogenesis, e.g., in the development of glioma [29]. Moreover, (Pyr^1)apelin-13 and apelin-13 are capable of increasing intracellular calcium concentrations in cultured human NT2-N neurons [24], as well as in HEK293 cells that have been stably transfected with the human apelin receptor [30].

Interestingly, apelin signaling is apparently not mediated by G protein βγ subunits and is Ras independent, but protein kinase C (PKC) dependent [27]. In accordance with this transduction mechanism, Szokodi and colleagues found that the inotropic effect of apelin in isolated rat hearts is markedly attenuated by inhibitors of PKC or the Na^+/H^+ exchanger [31]. Furthermore, it was shown that apelin-13 can phosphorylate p70S6 kinase at different residues in a time-dependent manner, involving either the phosphatidylinositol-3 kinase (PI3K) pathway that contributes to the control of endothelial cell proliferation or the ERK pathway in CHO and human umbilical vein endothelial cells [32].

Activation of PI3K and its downstream target Akt can lead to vascular relaxation, which is mediated in part by endothelial cell production of NO. As described in section "Apelin Expression in Rodent and Human Adult Tissue," apelin immunoreactivity was detected within the endothelia of small arteries. Interestingly, in rats the mean arterial pressure could be reduced after administration of apelin-12, apelin-13, and apelin-36, but in the presence of a NO synthase inhibitor that prevents the release of NO, the blood pressure lowering effect of apelin was abolished [17]. After administration of apelin-12 an elevation of the plasma nitrite/nitrate concentration was observed, suggesting that apelin activates the endothelial NO synthase, thus inducing

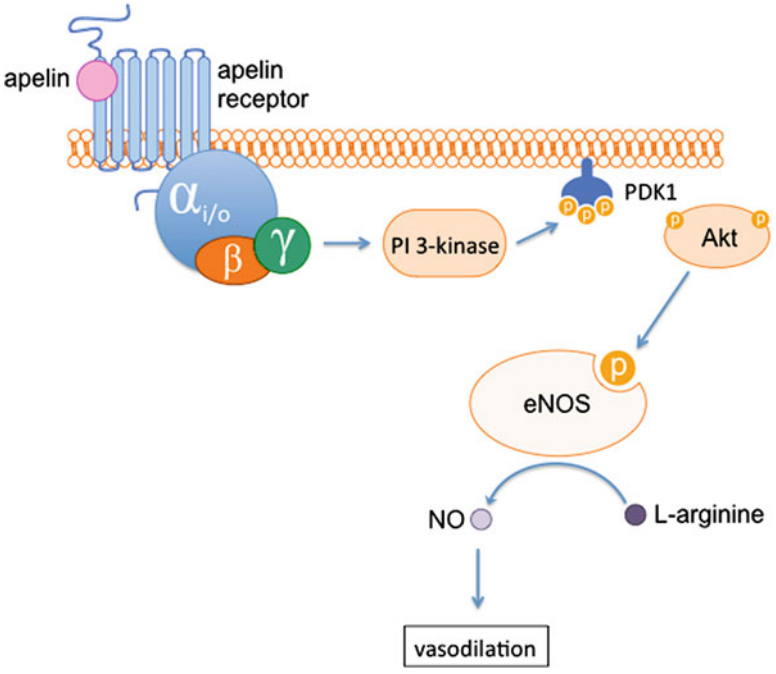

Fig. 7.1 Apelin binding to the apelin receptor initiates phosphatidylinositol-3 kinase (PI3K) activation through the $G\alpha_{i/o}$ subunit. Subsequent phosphorylation of 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt can activate the endothelial nitric oxide synthase (eNOS), leading to NO-induced vasodilation

relaxation of smooth muscle cells and lowering of blood pressure [17], most likely through phosphorylation of Akt by apelin (Fig. 7.1), as shown in CHO cells [32].

An alternative way of apelin receptor signaling is taking place via recruitment of β-arrestin following apelin receptor internalization [33, 34] in a clathrin-dependent manner [35]. Depending on the different apelin isoform that binds to the receptor, the apelin receptor is quickly recycled after internalization or trafficked to intracellular compartments, such as lysosomes. Also nonligand induced stretch-dependent activation and internalization was observed being mediated by β-arrestin binding with no G-protein activation involved [36]. Finally, the apelin receptor can form heterodimers with other GPCRs, e.g., AT1R [37], k-opioid receptor [38], neurotensin receptor 1 [39], and bradykinin 1 receptor [40], modulating its physiological role. In the case of apelin receptor–AT1R interaction apelin binding seems to blunt AT1R signaling [37].

In summary, apelin is able to activate diverse signaling proteins and cascades (see Chapman et al. [41] for more details) opening up many possibilities for cross-talk also to receptor tyrosine kinases, which are important in vascular development as well as tumorigenesis.

(Patho)physiological Role of Apelin Signaling in Mouse and Man

Since their discovery, both apelin and the apelin receptor have been shown to be expressed throughout the human body. Correspondingly, the peptide and its receptor have been associated with a range of physiological functions throughout the last decade. The structural relationship of the apelin receptor with AT1R [1] indicated that apelin could regulate cardiovascular functions in a comparable way like angiotensin II. Due to a strong apelin and apelin receptor expression in the healthy human heart and plasma [42], a role of apelin in human cardiac function was expected. Indeed, infusion of different apelin isoforms induced a dose-dependent positive inotropic effect in isolated rat hearts potently enhancing cardiac contractility [31]. In pathophysiological conditions like chronic heart failure due to coronary heart disease or idiopathic dilated cardiomyopathy, apelin mRNA levels were elevated in the left ventricle of such patients [42]. Moreover, it was reported that apelin plasma levels strongly increase in early heart failure and that levels of apelin receptor are high in late stage of the disease [43], suggesting that apelin signaling supports contractility of the failing heart in mild to moderate left ventricular dysfunction.

Counterintuitive, in apelin-receptor deficient mice an increased vasopressor response to angiotensin II was observed, indicating a counterregulatory role of apelin to that of angiotensin [44]. Indeed, intravenous injection of apelin was shown to lower systolic and diastolic blood pressure [14, 17] and also the risk for abdominal aortic aneurysm formation due to its peripheral antiatherogenic properties [45]. Also, findings of Kojima et al. showed apelin knockout mice with

decreased neointima formation in a carotid ligation model, implying that activation of the apelin receptor is detrimental in atherosclerosis [46]. That apelin expression is related to systolic blood pressure is further shown as circulating levels of apelin-12 were significantly lower in patients with essential hypertension [47]. It seems that apelin is a potent endothelium-dependent vasodilator, but can also elicit direct vasoconstrictor actions in both the artery and vein after removal of the endothelium [48]. Discrepancies in the literature concerning apelin receptor signaling as pressor or depressor might be a reflection of different apelin isoforms that have been used in these studies.

Double mutant mice, lacking both apelin receptor and AT1R, have a higher baseline blood pressure than mice lacking AT1R only, suggesting again a counterregulatory role of apelin signaling to that of angiotensin in blood pressure regulation [44]. Interestingly, central administration of (Pyr^{1})apelin-13 in rats caused an increase in arterial blood pressure and heart rate, which were even greater than after intravenous injection of (Pyr^{1})apelin-13 [47, 49], indicating that apelin is probably even more important in central than in peripheral regulation of the cardiovascular system. Therefore, (patho)physiological consequences of apelin signaling on the cardiovascular system remain to be clarified.

As mentioned in sections "Apelin Expression in Rodent and Human Adult Tissue" and "Apelin Receptor Expression," intense labeling of apelin and apelin receptor by in situ hybridization was also observed in the supraoptic and paraventricular nucleus of the hypothalamus. Interestingly, these regions are involved in the control of food and water intake, implicating a role of the apelin receptor system in body fluid balance [7, 9, 14, 21]. Similarly to the described cardiovascular functions, also regarding fluid homeostasis, opposite behavioral readouts on apelin actions have been reported. Taheri et al. observed an increase of water intake after central administration of apelin-13 in rats [50], whereas other studies found a decrease of water intake [51] or even no change in drinking behavior after central (Pyr^{1})apelin-13 injection [52]. Regarding feeding behavior, intraventricular apelin-12 injection showed reduced nocturnal food intake, while day-time injections stimulated feeding [53] and central apelin-13 injection caused a reduction in food intake in both fed and fasted rats [54]. Interestingly, central apelin-13 administration did not change food or water intake in rats fed a high-fat diet, but decreased the central expression of the apelin receptor, suggesting an apelin insensitivity that could eventually promote obesity [51].

Abnormal fluid balance manifested also in apelin receptor knockout mice, which showed decreased drinking behavior but no reduced urine volume and increased osmolality after water deprivation for 24 h as observed in wild-type mice, symptomatic of an antidiuretic effect of apelin in vivo [55]. However, in lactating rats, intravenous injection of apelin-17 induced diuresis [56]. In humans, acute osmotic stimuli (hypertonic saline infusion vs. water loading) induced reciprocal regulation of plasma apelin and vasopressin concentrations with increased plasma apelin levels and decreased vasopressin levels under water loading conditions [57]. In rodent studies, counteracting vasopressin actions of apelin

by inhibiting vasopressinergic neuron activity and systemic vasopressin secretion from the hypothalamo–neurohypophysial system were also described [20].

Recent reports connected central nervous system expression of apelin to a neuroendocrine function as apelin pretreatment was shown to be neuroprotective for hippocampal neurons after NMDA-receptor-mediated excitotoxic injury [58]. Moreover, apelin-13 prevented apoptosis in primary mouse cortical neurons through reduced reactive oxygen species generation and mitochondrial dysfunction [59]. The neuroprotective action of apelin and its receptor are most likely mediated via the phosphorylation of Akt and ERK1/2 [60].

That apelin might be involved in the regulation of the immune system was first described by Habata and colleagues in 1999 who showed that apelin can partially suppress cytokine production from mouse spleen cells activated by CD3 cross-linking [16]. Apelin was also found to inhibit lymphocytic cholinergic activity during immunological responses by downregulating choline acetyltransferase mRNA expression and activity via the apelin receptor [61]. Furthermore, apelin acts immunoprotective in aortic aneurysm formation through limiting proinflammatory macrophage burden and lowering the expression of interleukin-6 and tumor necrosis factor α in the vessel wall [45]. Thus, apelin seems to play a role in the modulation of the immune system, but the consequences of the described effects need to be further elucidated.

Apelin Signaling in Cardiovascular Formation

Apelin receptor expression during embryonic vascular development is known since 1996 [2] (see also section “Identification of the Apelin Receptor and Its Endogenous Ligand Apelin”). The localized expression of apelin to the leading edge of apelin receptor expressing retinal vessel cells and the structural relationship of the apelin receptor to CXC chemokine receptors [2, 6] suggested a chemotactic function of apelin signaling on endothelial cells [19]. In our own work from 2007 [4], we thus focused on the developmental aspects of apelin and its receptor and unambiguously demonstrated that embryonic apelin expression has remained conserved during vertebrate evolution and is closely associated with the initiation of angiogenic blood vessel growth in the frog *Xenopus laevis* as well as the mouse [4]. In vitro, apelin was able to promote chemotaxis of mouse retinal as well as brain endothelial cells and in our hands on primary human endothelial cells [4, 62, 63]. The functional importance of apelin signaling in angiogenesis and its epistatic relationship with vascular endothelial growth factor (VEGF) signaling was established in a series of loss- and gain-of-function experiments in *Xenopus* tadpoles by us and others simultaneously, confirming that apelin signaling is necessary and sufficient to promote angiogenic sprouting of intersomitic blood vessels in vivo [4, 63]. Apelin appears to act first in a paracrine fashion to induce intersomitic blood vessel outgrowth shaping the primitive vascular plexus, while then it possibly serves for vessel guidance and maturation via the angiogenic tip cells where endothelial apelin gets localized to [4, 64–67].

In the meanwhile, mild vascular effects were observed in apelin knockout mice and described as narrow blood vessel phenotype, while apelin overexpressing mice had enlarged but stable vessels with reduced vascular permeability [66, 68]. Later, reassessment of apelin knockout mice showed retardation of retinal vascular development and apelin seemed to be a prerequisite factor for hypoxia-induced angiogenesis [63, 69]. Recently, Liu et al. impressively showed that Apln-CreER mice can be utilized as a novel tool to distinguish sprouting endothelium from stabilized vasculature in various pathological contexts, such as ischemia or tumor development [70]. Furthermore, McKenzie et al. demonstrated that mouse apelin is important for non-neovascular remodeling of the retina [71], while in zebrafish, hypoxia-induced apelin expression stimulated regenerative angiogenesis in the dorsal fin [72]. In regenerative angiogenesis after myocardial infarction, apelin signaling served as a chemoattractant for circulating endothelial cell progenitors [73].

In the development of the heart, the apelin receptor pathway seems to be responsible for myocardial cell specification [74–78]. While apelin receptor mutant mice are born at submendelian ratios, apelin knockout mice are viable and fertile [68, 74, 79]. The lethality observed in apelin receptor knockout mice is due to growth retardations and cardiac malformations including a markedly deformed vasculature of the yolk sac at embryonic day (E)10.5 [80]. Many of the dissected embryos at E10.5 had enlarged and aberrantly formed hearts. Embryos surviving until E15.5 had, e.g., thinned myocardia and ventricular septal defects [81]. Surprisingly, apelin receptor deficient mice did not show gross defects if they developed to adulthood, except from mild cardiovascular pathologies, including modest decrements in cardiac contractility under basal conditions and strikingly reduced exercise capacity under exercise stress [80]. A possible explanation for this discrepancy in ligand and receptor knockout phenotype was recently found by the identification of a second ligand called apelin receptor early endogenous ligand (apela, also called toddler or elabela). Apela mutant zebrafish exhibit severe developmental defects including absence of the developing heart phenocopying the loss of the apelin receptor resulting in embryonic lethality due to cardiac malformations in mice [80]. Early ectopic expression of apelin was able to rescue the embryonic defects of apela mutant zebrafish embryos suggesting that apela and apelin both signal through the apelin receptor [82, 83].

In 2015, Kidoya et al. reported that arterial-venous alignment in the skin is determined by apelin receptor expression in venous endothelial cells receiving apelin signals from arterial endothelial cells later during embryogenesis [84]. They also showed that apelin- and apelin receptor-deficient mice exhibit arterial-venous disorganization, which results in a defect in thermoregulation in these mice [84]. In summary, the emerging picture of apelin signaling cascades (Fig. 7.2) opens a lot of possibilities for the ligand and its GPCR to serve as a future cardiovascular-centered therapeutic target.

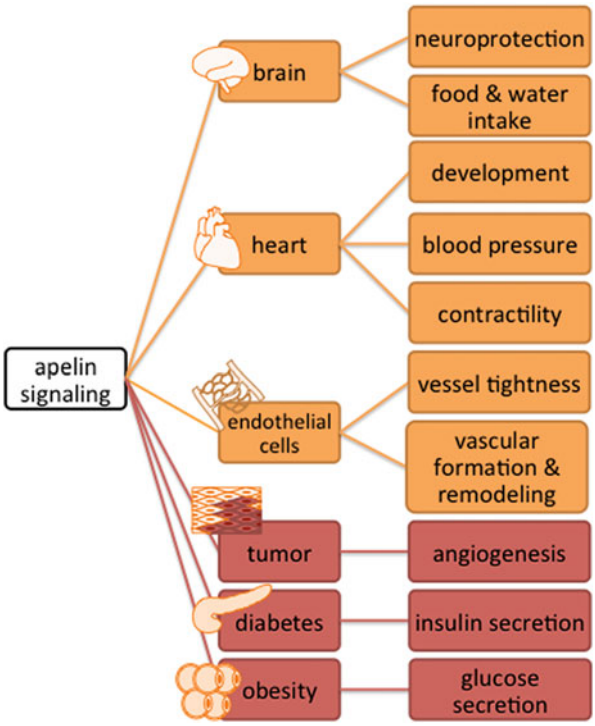

Fig. 7.2 Central and peripheral apelin and its receptor exert a wide variety of physiological (*bright*) and pathophysiological (*dark*) functions. Ligand and receptor are broadly expressed in peripheral tissues (e.g., adipose tissue, cardiovascular system) and in the central nervous system

Apelin in Metabolic Disorders of Mouse and Man

Similar to other adipokines, apelin is expressed in preadipocytes and its expression increases during differentiation to adipocytes [85]. The apelin receptor is also found in adipose tissue, including adipocytes and the stromal vascular fraction [85, 86]. Apelin serum levels are related to the nutritional status in rodents and humans. For example, wild-type mice that have been on a calorie-rich diet for 8 weeks with consequent hyperglycemic and hyperinsulinemic states presented increased adipocyte apelin expression and apelin plasma levels [85]. In the same study by Boucher et al., a period of 24 h fasting significantly decreased adipocyte apelin expression and blood plasma levels, which got back to initial levels after refeeding the animals [85].

Apelin-13, apelin-17, and (Pyr^{1})apelin-13 isoforms can also be found in human plasma [57], where their levels correlate with the nutritional status. While obese and hyperinsulinemic patients show higher apelin serum concentrations [85, 87, 88], anorectic girls have decreased serum levels of apelin as a result of limited fat tissue in these patients [89]. However, not the body composition itself, but a close relationship

between apelin and pancreas-derived insulin seems to be the main driver for apelin secretion, as for example normal-weight type 2 diabetic (T2D) patients show increased plasma apelin levels [90] and morbid obese patients suffering also from T2D have increased plasma apelin levels whereas morbid obese patients without T2D have similar levels compared to normal weight controls [91]. Plasma apelin receptor levels are higher in obese individuals, but after body weight reduction due to caloric restriction, apelin receptor levels could be reversed [92]. Krist and colleagues analyzed apelin plasma levels of a cohort of individuals in three independent intervention studies, including bariatric surgery, exercise, and hypocaloric diet [93]. In all groups, they found a correlation between decreased apelin serum levels and improved insulin sensitivity that where independent of body weight, suggesting that insulin is the main driver of apelin expression and not the obesity state per se, which is attributed with bigger adipocyte size. In line with this theory, plasma insulin levels correlate with adipocyte apelin expression in leptin receptor deficient db/db mice [85]. Apelin deficient mice, that received exogenously delivered apelin for several weeks performed better in insulin and glucose tolerance tests. Also administration of apelin to obese diabetic db/db mice improved insulin sensitivity [94]. Moreover, data of Dray et al. show that in chow-fed mice, acute intravenous injection of apelin has a powerful glucose-lowering effect by stimulating glucose uptake in skeletal muscle and adipose tissue [95], underlining a relevant role of apelin in glucose metabolism. In hyperglycemic and hyperinsulinemic mice, glucose tolerance could be largely restored through administration of apelin [95], while in human adipocyte tissue explants apelin expression was increased after insulin treatment [85]. A recent report using pancreatic islet specific apelin receptor knockout mice confirmed the stimulatory role of apelin signaling in the regulation of pancreatic islet homeostasis and in metabolically induced ß-cell hyperplasia [96].

In a mouse model of type 1 diabetes, which is characterized by the lack of insulin-producing ß-cells, isolated adipocytes from streptozotocin-treated mice showed decreased apelin mRNA levels [85]. Interestingly, basal apelin gene expression seems to be fat depot specific, as apelin transcripts were lower in subcutaneous white adipose tissue compared to epididymal or retroperitoneal white adipose tissue, similar to adiponectin or leptin [97]. Furthermore, Than and colleagues recently demonstrated that apelin signaling is involved in browning of white adipose tissue [98]. The authors could show that after (Pyr^{1})apelin-13 treatment, brown-like characteristics, including increased uncoupling protein (UCP) 1 expression and basal mitochondrial respiration, were increased in human white adipocytes. In addition, higher basal activity of brown adipocytes and the initiation of brown adipocyte differentiation were also stimulated by exogenous (Pyr^{1})apelin-13 application through PI3K/Akt and AMP-activated protein kinase (AMPK) signaling pathways [98].

The metabolic syndrome usually goes along with the massive accumulation of triglycerides in the liver leading to hepatic steatosis. Apelin and apelin receptor mRNA levels were reported to be higher in rats with liver cirrhosis than in controls. Human patients with marked hepatic failure had elevated circulating apelin levels and most interestingly, apelin levels gradually increased in higher grades of hepatocellular carcinoma. Chronic blockage of the apelin receptor in rats with cirrhosis

diminished hepatic fibrosis and decreased vessel density [99, 100], suggesting important roles of apelin signaling in liver diseases.

In summary, the described human and rodent data indicate that apelin and its receptor influence peripheral and central energy metabolisms and may contribute to obesity and related metabolic diseases, such as T2D. However, the exact mechanisms how apelin and its receptor affect, e.g., glucose metabolism are still unclear and human data to confirm the homeostatic functions of apelin are still lacking.

Apelin Signaling in Tumor Development

One of the hallmarks of multistep tumorigenesis is the ability of tumor cells to stimulate the vasculature to form new blood vessels in a process termed tumor angiogenesis. The resulting vessels are structurally and functionally abnormal and contribute to a hostile microenvironment (low oxygen tension and high interstitial fluid pressure), which in turn selects for a more malignant phenotype of the tumor with increased morbidity and mortality [101]. Moreover, tumor vessels show an imbalanced and local overexpression of cytokines, particularly of hypoxia-regulated VEGF, which is a major permeability and proangiogenic factor that is usually highly expressed in tumors [102].

By in situ hybridization on patient specimens of brain tumors, specifically glioblastoma multiforme (GBM, world health organization [WHO] grade IV [103]—belonging to the most aggressive neoplasms in humans [104] offering patients an average survival time of approximately 1 year after diagnosis at best), we found the first hint for a role of apelin signaling in tumor development. While no apelin and only low levels of apelin receptor were found in normal brain vessels, we detected a dramatic upregulation of apelin and its receptor expression within GBM-associated microvascular proliferations, particularly in areas of vessel sprouting and branching [4]. Coexpression of ligand and receptor in the tumor vasculature suggests an autocrine mode of signaling, similar to the developmental regulation we have described [4]. In addition, we also found apelin expression in the radially orientated neoplastic cells surrounding band-like necrosis (Fig. 7.3). In this hypoxic area that can be marked by in situ hybridization against VEGF [105], apelin is coexpressed with VEGF suggesting a collaborative function of apelin and VEGF paracrine signaling from tumor to endothelial cells in GBM angiogenesis.

In line with the role of apelin in tumor angiogenesis are the collective findings that the apelin gene sequence is containing hypoxia responsive elements and can thus, likewise to VEGF, be activated by hypoxia-inducible factor (HIF)1α upregulation in the tumor microenvironment [63, 79, 106, 107].

Independent evidence for a role of apelin in gliomagenesis comes from the identification of a novel glioma-suppressor gene named hHSS1 [108]. Interestingly, overexpression of hHSS1 in human U87 glioma cells showed downregulation of a set of genes by microarray expression profiling where the most prominently regulated gene was apelin [108].

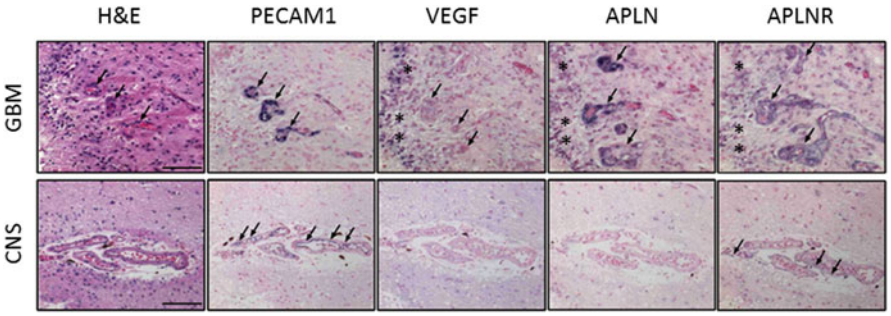

Fig. 7.3 Apelin and its receptor are upregulated in GBM. GBM specimens and normal central nervous system (CNS) tissue were stained by in situ hybridization. Glomeruloid tuft-like microvascular proliferations (*arrows*) are positive for endothelial cell marker PECAM1, apelin and apelin receptor. Radially oriented neoplastic cells surrounding band-like necrosis (*asterisks*) express VEGF and to some extent apelin and apelin receptor. PECAM1 and moderate apelin receptor expression is detected in vessels within normal CNS tissue. Scale bar 100 μm; figure adapted from figure 2 in Kälin et al. [4] reprinted with permission from Elsevier

A comparison of gene expression profiles in tumor versus normal endothelium also identified apelin as a tumor endothelial specific gene [109]. Additionally, in human breast carcinoma, apelin expression was detected in the tumor vessels by immunohistochemical analysis [110]. Recent work from Masiero et al. further provides important insights into the regulation of tumor angiogenesis and antiangiogenic tumor resistance [111]. The authors analyzed the expression profile of more than 1000 primary human cancers (among them 121 head and neck squamous cell carcinomas, 959 breast cancers, and 170 clear cell renal cell carcinomas) to generate a vascular/angiogenesis core signature composed of genes whose expression correlates with that of several well-recognized angiogenesis "seed" genes in multiple cancers. To identify novel genes involved in angiogenesis, this analysis was complemented by in vivo expression profiling of antiangiogenic therapies of the top-ranked candidates. Interestingly, the apelin receptor belonged to this tumor angiogenesis core signature [111].

Furthermore, in studies analyzing patient samples of nonsmall cell lung cancer, high apelin expression was associated with elevated microvessel density and poor overall survival [112] and apelin was also found to be overexpressed in patient specimen with oral squamous cell carcinoma [106].

Using mouse models, Sorli et al. showed that artificial ectopic overexpression of apelin in subcutaneous tumor implants leads to increased intratumoral vessel formation and enhanced tumor growth [113]. In contrast to these results, Kidoya and colleagues elaborated on a role for apelin and its receptor in tumor vessel maturation, instead of sprouting tumor angiogenesis [114]. They demonstrated that ectopic apelin overexpression in subcutaneously implanted murine colon and lung cancer cells led to bigger tumor vessels but no increase in vessel density. Moreover, they show that apelin transfection in these subcutaneous tumor implants greatly inhibits their growth [114]. They speculate that apelin-induced normalization of the tumor vasculature

Table 7.1 The top ten annotation clusters identified by the DAVID Functional Annotation Clustering Tool for apelin-coregulated GBM genes with Pearson score starting at 0.25 (357 DAVID IDs)

Annotation cluster	Representive annotation term	Enrichment score
1	Angiogenesis	5.96
2	Glycolysis	4.18
3	Signal	4.00
4	Regulation of cell motion and migration	3.68
5	Cell adhesion	3.18
6	Extracellular matrix	2.72
7	Tube morphogenesis	2.67
8	Vasodilation/regulation of blood vessel size	2.64
9	Blood coagulation and wound healing	2.54
10	EGF and EGF-like /Notch signaling	2.42

may suppress tumor growth and could be exploited to improve efficacy of conventional tumor therapy. Alternatively, vascular apelin expression could be used to target drug delivery. As a proof of concept, Kawahara and colleagues show that apelin-conjugated liposomes end up more efficiently in endothelial cells of NIH-3T3 tumor cells [115].

We have interrogated the cancer genome atlas (TCGA) provisional data set of GBM tumors using the cBioPortal for genes being coexpressed with apelin. Interestingly, in GBM tumor samples that show apelin upregulation, the gene clusters with the highest enrichment scores fall into biological functional classes for angiogenesis and vascular rearrangement (Table 7.1).

The role for angiogenesis in providing oxygen and nutrients for tumor growth makes it an attractive target for potential therapy [116]. Thus, the humanized anti-VEGF monoclonal antibody bevacizumab had been approved for multiple oncological indications. Unfortunately, monotherapy with antiangiogenic agents has not been as effective as hoped for [117]. Two randomized phase III trials—AVAglio and RTOG-0825—investigated the addition of bevacizumab to standard-of-care therapy in newly diagnosed glioblastoma patients [118, 119]. However, the increased progression-free survival did not translate into an expected overall survival benefit in either study [120]. In GBM such treatment may fail due to the release of alternative angiogenic factors [121] or due to increased tumor cell invasion and increased vessel cooption [122, 123]. Moreover, antiangiogenic therapy results in endothelial cell apoptosis, vascular thrombosis, loss of microvascular integrity, and vascular regression, which are of limited duration only. It has become apparent that the tumor microvascular system is capable of adapting to the described antivascular stress, thus inducing a reactive resistance to antiangiogenic therapy [124]. This resistance is based on an increased expression of proangiogenic molecules and a recruitment of vascular support cells. So far, the molecular mechanisms remain not well understood and thus the exploration of novel angiogenic pathways, such as apelin signaling, is of foremost importance.

Metabolic Changes in Brain Tumor

Glioma are the most frequent primary tumors of the brain. Regardless of aggressive surgery, radio- and chemotherapy, and treatment with DNA alkylation agents, malignant glioma remain uniformly fatal in patients. There is currently little known about the mechanisms that help these tumor cells to stay untouched from standard postsurgical treatment paradigms. However, it can be assumed that their pathological function is highly supported by the activity of parenchymal cells that form the tumor's stromal niche, e.g., endothelia, astrocytes, intrinsic neural precursor cells, and microglia [125–127]. As VEGF-centered antiangiogenesis was not successful for glioblastoma intervention (see section "Apelin Signaling in Tumor Development"), tumor metabolism comes back into focus.

In the past years, more and more cancer-associated mutations in metabolic enzymes were recognized. In glioma mutations, two isoforms of isocytrate dehydrogenase (IDH), IDH1 and IDH2 have been described. It was found that high-grade glioma containing IDH mutations showed a more favorable prognosis with a median survival that was approximately doubled [128, 129]. The reason for that observation is under intense investigation. IDH1 and IDH2 couple the reversible conversion of isocitrate to α-ketoglutarate and $NADP^+$ to NADPH [130]. The IDH1 R132 mutation leads to accumulation of 2-hydroxyglutarate, which is structurally similar to α-ketoglutarate and may competitively inhibit prolyl hydroxlyase, which targets HIF1a for proteasomal degradation [131]. In addition, the 2-hydroxyglutarate production by the IDH-mutant cells seemed to inhibit histone demethylation leading to a block of differentiation of precursor cells [132]. Thus, metabolic changes in IDH mutant brain tumors are able to transform brain tumor cells stimulating invasion, cell survival, and angiogenesis [133, 134].

In order to support fast proliferation, cancer cells alter their metabolism generating lactate from pyruvate by aerobic glycolysis, called the Warburg effect [135]. Although this mechanism costs more energy in terms of ATP consumption and glucose uptake, it allows the tumor cells to obtain the required building blocks for the synthesis of nucleotides, fatty acids, and amino acids for rapid growth at low oxygen consumption [136]. Also in GBM glycolysis is upregulated when compared to normal brain tissue [137, 138]. Interestingly, genes coregulated with apelin expression in GBM also fall into the functional class for glycolysis (Table 7.1).

The increase in glucose uptake is used in clinics to image high-grade glioma by FDG-PET (fluorodeoxyglucose-positron emission tomography). However, as high glucose consumption in normal brains is producing high background, novel PET approaches using amino acid metabolites are being tested and seem to produce better results with higher contrast enhancement [reviewed in 139]. New approaches in MRI (magnetic resonance imaging) might also allow the monitoring of the conversion of pyruvate to lactate or even IDH status by measuring α-ketoglutarate levels [140, 141].

Interestingly, also endothelial cells enhance glycolysis during angiogenic sprouting. They upregulate glucose transporter (GLUT-1), lactate dehydrogenase-A (LDH-A), and 6-phosphopfructo-2-kinase/fructose-2,6-bispohosphatease-3 (PFKFB3) [142–145] and PFKFB3 depletion was shown to impede vessel sprouting blocking

migration of tip cells and proliferation of stalk cells [142]. Likewise to the tumor cells, aerobic glycolysis might help the endothelial tip cells to funnel excess metabolites into the pentose phosphate pathway, thus supporting cell proliferation and migration. Due to the low oxygen dependence during aerobic glycolysis, endothelial cells might be prepared to sprout into hypoxic areas of the tumor. Thus, apelin signaling might be an ideal target to attack tumor metabolism and angiogenesis in a synergistic fashion.

Apelin Signaling in Cancer Metabolism

The concept that tumor metabolism is influenced by systemic metabolic states came up in many epidemiological studies showing a correlation between metabolic imbalance, as seen during obesity and diabetes, and cancer progression [146]. Inflammatory processes taking place in the central nervous system, where they are mediated by, e.g., resident microglia and infiltrating peripheral macrophages, have been recently positively correlated with obesity and obesity-associated diseases [147], fueling the idea that tumor metabolism is also influenced by systemic metabolic states. For example, a strong expression of leptin in ductal mammary tumors was associated to be rather procarcinogenic, while low levels of adiponectin was shown to have anti-carcinogenic effects in breast cancer development [148, 149].

Apelin and its receptor were shown to be overexpressed in different types of cancer, including hepatocellular carcinoma [150], oral cell carcinoma [106], non-small cell lung carcinoma [112], colon or lung adenocarcinoma [151, 152], and in chronic hepatitis C patients with late liver fibrosis or cirrhosis [100]. In 2015, Lacquaniti et al. found that apelin can be a useful biomarker for evaluating cancer diseases progression [153], because they showed that serum apelin levels increase gradually correlating to cancer stage and thus decreased patient survival. Furthermore, Berta et al. had already shown that hypoxia-induced upregulation of apelin was associated with a poor prognosis in oral squamous cell carcinoma patients [112] and in glioma, apelin expression is highest in grade IV glioblastoma correlating with poor survival (own unpublished data). Interestingly, Lacquaniti et al. show a strong correlation between apelin and nutritional parameters, such as body weight and body mass index, connecting apelin and cancer cachexia [153]. Increased apelin serum levels were also found in patients with gastroesophageal cancer, which were even more elevated in cachectic patients [154]. In contrast, apelin serum concentrations were shown to be higher in women with endometrial cancer and this increase significantly correlated with the obesity state and fasting insulin levels of the female patients [155], indicating that apelin serum levels and their influence on cancer progression are critically determined by the type of cancer analyzed.

Endometrial cancer is known to be associated with increased insulin resistance and obesity, but in the study by Altinkaya et al. apelin levels did not inversely correlate with cancer stage [155], exemplifying that tumor angiogenesis is not the only source of apelin upregulation and that apelin levels may be influenced by multiple circulating factors in the serum, such as leptin, insulin, hormones, and

inflammatory cytokines. Thus in tumor development, apelin may on the one hand act as an angiogenic factor [4, 113, 156] and on the other hand as an inhibitor of insulin secretion and glucose tolerance [85, 96, 157, 158].

As reviewed in section "Apelin in Metabolic Disorders of Mouse and Man", apelin serum levels are correlated to the nutritional status in mice and man. In vitro, it was shown that apelin can stimulate glucose transport in muscle cells in addition to insulin [95, 159]. In vivo, apelin was able to increase myocardial glucose uptake and glucose transporter 4 (GLUT4) membrane translocation [160]. Oral application of (Pyr^{1})apelin-13 peptide leads to increased glucose transporter 2 (GLUT2) levels due to AMPK activation in enterocytes [161]. However, it remains still unclear if apelin levels can also influence whole-body glucose consumption.

As apelin was identified as tip cell marker during embryogenesis [67] where it can serve as a paracrine factor for chemoattraction of the sprouting vessels, the mentioned observations raise the possibility that apelin tip cell expression might be involved in metabolic regulation of vessel sprouting. Moreover, increased serum levels of apelin in obese patients might support tumor growth by acting as a paracrine factor to enhance tumor angiogenesis (Fig. 7.4).

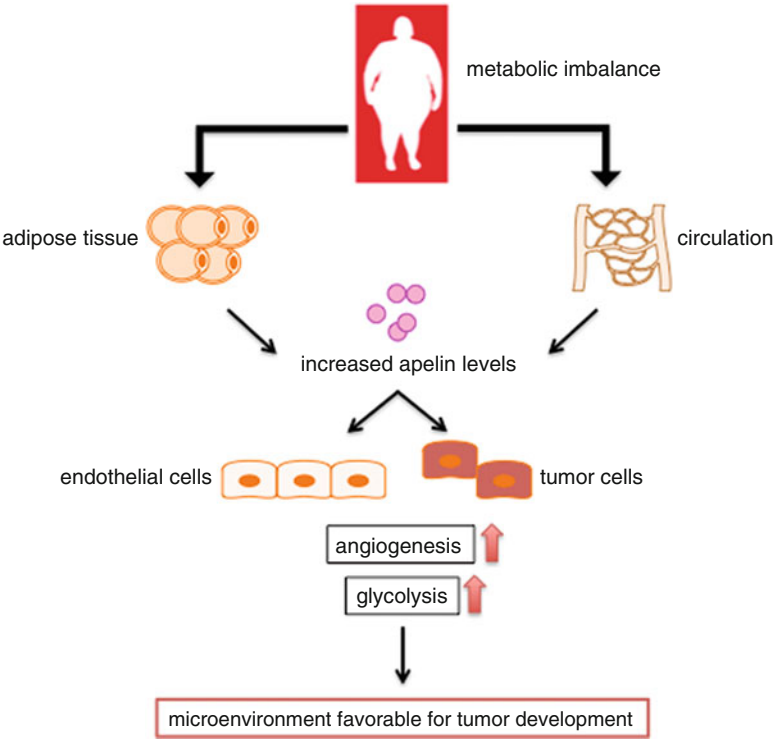

Fig. 7.4 Overnutrition results in increased release of apelin from adipocytes and higher circulating levels of apelin, leading to a wide variety of responses in endothelial cells and tumor cells, which possibly primes the microenvironment to support tumor development and/or progression

Next to glycolysis, also β-oxidation represents a potent energy source and first effects of apelin on lipid metabolism were described. For example, chronic apelin peptide treatment in chow or high-fat diet fed mice led to a decrease in weight and triglyceride content of white adipose tissue without affecting food intake, while increasing the thermogenic marker UCP1 and the regulator of fatty acid export UCP3 in brown adipose tissue [162]. Similar results were found in apelin overexpressing mice fed a calorie-rich diet [163]. Moreover, fatty acid oxidation in muscles was improved by AMPK-dependent mechanism in obese and insulin-resistant mice treated with apelin-13 [164]. If apelin can directly act on glucose metabolism and tricarboxylic acid cycle in tumor cells and sprouting endothelia needs to be investigated.

As reviewed in this chapter, excessive adipose tissue accumulation followed by expression changes in the adipokine apelin can be associated with increased cancer risk, most likely through elevated inflammation, decreased apoptosis, and effects on endothelial cell proliferation and migration, and possibly through direct actions on tumor and endothelia cell metabolism (Fig. 7.4).

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Chapter 8
Novel Adipocytokines: Monocyte Chemotactic Protein-1, Plasminogen Activator Inhibitor-1, Chemerin

E. Angela Murphy

Abstract Given the global increase in obesity, significant research has focused on understanding the role of adipokines, or adipocytokines, on obesity-related pathologies. In the last two decades, hundreds of adipokines have been acknowledged. Monocyte chemoattractant protein (MCP) 1, chemerin, and plasminogen activator inhibitor (PAI) 1 are three such signaling molecules that have recently been classified as adipokines. Although these novel adipokines have clear roles in activities independent of obesity-related pathologies, emerging evidence now identifies their importance in metabolic-related diseases and cancer. This chapter will first provide a brief discussion on the discovery, structure, and receptors for MCP-1, chemerin, and PAI-1. Second, we will discuss the role of these novel adipocytokines on adiposity and subsequent obesity-related diseases. Finally, we will examine the available literature linking MCP-1, chemerin, and PAI-1 to tumorigenesis. Based on the literature, it is clear that these novel adipokines can impact disease pathology as related to obesity and tumorigenesis. The majority of the literature links increased expression of these adipokines to disease pathology. However, there is also evidence implicating that a decrease in these factors can influence obesity and cancer. Thus, it is possible that the role for these adipokines may be dependent on the model. Further, it is likely that the dysregulation of these novel adipokines resulting in either overexpression or underexpression results in an unfavorable outcome.

Keywords Monocyte chemoattractant protein 1 • Chemerin, plasminogen activator inhibitor 1 • Obesity • Tumorigenesis

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Introduction

Over the last several decades, we have observed a worldwide increase in obesity and subsequent related chronic diseases like cancer. The widespread consumption of energy dense foods and the lack of physical activity are mostly to blame for this phenomenon. As a result, a significant amount of research has been dedicated to understanding the mechanisms linking obesity to disease pathology. One such area includes the investigation of adipocytes. Adipokines, or adipocytokines, are cytokines produced in the adipose tissue. They have a wide range of activities and can include inflammatory mediators, angiogenic proteins, and metabolic regulators. Some adipokines are produced primarily by the adipose tissue themselves while others are also synthesized in other tissues. Adipokines can act locally or peripherally and are thought to influence a number of processes including insulin sensitivity, adipocyte differentiation, and glucose and fatty acid metabolism. As such, they have been linked to a number of chronic diseases including cancer.

Leptin, produced exclusively by adipocytes, was the first adipokine to be discovered in 1994. Since then hundreds of these adipocytokines have been acknowledged. Monocyte chemoattractant protein (MCP) 1, chemerin, and plasminogen activator inhibitor (PAI) 1 have recently been classified as adipokines. While these adipokines are not produced exclusively by adipose tissue and it is well established that they have clear roles in activities independent of obesity-related pathologies, emerging evidence now highlights their importance in metabolic-related diseases and cancer. This chapter will first provide a brief discussion on the discovery, structure, and receptors for MCP-1, chemerin, and PAI-1, which is important to understanding their role in disease mechanisms. Second, we will discuss the role of these novel adipocytokines on adiposity and subsequent obesity-related diseases. Finally, we will examine the available literature linking MCP-1, chemerin, and PAI-1 to tumorigenesis.

Novel Adipokines: Discovery, Structure, and Receptors

Monocyte chemoattractant protein (MCP) 1, chemerin, and plasminogen activator inhibitor (PAI) 1 have recently been classified as adipokines. These novel adipokines have well-established roles in processes independent of obesity-related pathologies. However, emerging evidence identifies their importance in metabolic-related diseases and cancer. Here we will introduce MCP-1, chemerin, and PAI-1 and discuss their discovery, structure, and receptors, which is important to understanding the role of these adipokines in chronic disease risk.

Monocyte Chemotactic Protein-1

Discovered in 1989, monocyte chemoattractant protein -1 (MCP-1), also referred to as chemokine (CC-motif) ligand 2 (CCL2), is a potent monocyte chemoattractant [1]. It is a member of the CC subfamily of chemokines and is credited as being the first

identified human CC chemokine. The gene for human MCP-1 is located on chromosome 17 (chr 17, q11.2) and its expression can be induced by a number of mediators including various cytokines, growth factors, and interferons as well as bacterial lipopolysaccharide among others [2]. MCP-1 is produced as a protein precursor containing a signal peptide of 23 amino acids attached to a mature peptide of 76 amino acids [3, 4]. Human MCP-1 protein, also known as monocyte chemotactic and activating factor, has been reported to be identical to the mouse JE/MCP-1 gene product [5].

MCP-1 is arguably the most studied member of the chemokine family. Initial functional characterization of this chemokine was performed using purified protein under in vitro conditions. Subsequent targeted gene disruption to create MCP-1 deficient mice has allowed for the examination of the role of this chemokine in the recruitment of immune cells and subsequent disease pathology. MCP-1 is produced by an abundance of cell types including epithelial, smooth muscle, endothelial, fibroblasts, mesangial, monocytic, astrocytic, and microglial cells [6–8]. However, monocytes and macrophages are largely recognized as being the major sources of MCP-1 [3, 9]. MCP-1 is responsible for the regulation of migration and infiltration of monocytes, memory T lymphocytes, and natural killer cells to sites of infection, inflammation, or injury [10, 11].

MCP-1 exerts its effects through binding to G-protein-coupled receptors on the surface of leukocytes that have been targeted for activation and migration. These receptors, once activated, trigger a set of cellular reactions that result in inositol triphosphate formation, intracellular calcium release, and protein kinase C (PKC) activation [12]. Although MCP-1 binds to both CCR2 and CCR11 receptors, CCR2 is the classic MCP-1 receptor and largely responsible for its activity. In fact, binding to CCR11 does not result in increased intracellular calcium release, which is a necessary requirement for chemotaxis [13]. Further, MCP-1 has a lower affinity for CCR11 than other chemokines. In addition to CCR2 and CCR11, three other receptors have been shown to bind MCP-1 including the Duffy antigen receptor for chemokines (DARC), D6, and US28. Unlike CCR2, these receptors are not specific for MCP-1 but are known to bind other chemokines with similar affinity. It is unclear as to the role of DARC and D6 as no signaling has been observed following binding to MCP-1 [14]. On the other hand, US28 is a functional chemokine receptor and has been reported to accelerate inflammation in the presence of MCP-1 [15, 16].

Chemerin

Chemerin, also known as tazarotene-induced gene 2(TIG2) or retinoic acid receptor responder 2 (RARRES2) is a relatively recently discovered adipokine. The chemerin gene was actually originally identified in 1997 as a novel retinoid-responsive gene in psoriatic skin lesions [17]. In 2003, the chemerin protein was identified as a secreted ligand of the orphan G protein-coupled receptor chemokine-like receptor (CMKLR) 1, also known as chemR23 [18]. Since then, it has also been demonstrated to serve as a ligand for G protein-coupled receptor (GPR) 1 and chemokine

(C-C motif) receptor-like (CCRL) 2. However, to date, the interaction between chemerin and the CMKLR1 receptor is the most well studied. CMKLR1 is expressed on various immune cell populations including monocytes, macrophages, microglial cells, natural killer cells, and dendritic cells [19–21]. As such, chemerin functions as a chemoattractant that recruits these cells to the site of tissue injury or damage. Interestingly, B and T lymphocyte cell populations and granulocytes have been reported not to express CMKLR1. Less is known about the involvement of GPR1 and CCRL2 on chemerin bioactivity. Although GPR1 displays high affinity for chemerin, it signals poorly in classic G protein-mediated pathways [22]. Thus, it has been hypothesized that GPR1 may serve as a decoy receptor for chemerin. GPR1 is reported to not be expressed on immune cell populations but is expressed in the central nervous system, skeletal muscle, skin, and adipose tissue [22]. With regards to CCRL2, it is reportedly expressed on human immune cells including monoctyes, macrophages, dendritic cells, neutrophils, T cells, natural killer cells, and mast cells [23, 24]. CCRL2 is upregulated upon activation of immune cells by proinflammatory mediators including LPS and TNF-α. Like CMKLR1 and GPR1, CCRL2 binds chemerin with high affinity but this interaction does not appear to promote any signaling in the cells and does not result in CCRL2 internalization [22].

Structurally, human chemerin is translated as a 163 amino acid preproprotein that is secreted as a 143 proprotein following the proteolytic cleavage of a signal peptide [25]. This proprotein is known to have minimal chemotactic activity and requires further extracellular C-terminal processing [20, 25, 26]. The extent of C-terminal cleavage is dependent on the location from which chemerin is isolated. For example, chemerin purified from hemofiltrate lacks nine amino acid residues in the C-terminal region whereas serum-derived chemerin lacks only eight amino acid residues and chemerin in human ovary cancer ascetic fluids lacks only six C-terminal amino acids in comparison to its precursor [27, 28]. Proteolytic processing is also thought to be involved in the inactivation of chemerin. Thus, proteolytic processing of chemerin appears to be a key regulatory mechanism that determines the level of its bioactivity.

Plasminogen Activator Inhibitor 1

PAI-1, earlier also called the fast acting inhibitor, the endothelial cell inhibitor, and the beta-migrating inhibitor, was described in the late 1970s as a fibrinolytic inhibitor [29]. PAI-1 belongs to the serine protease inhibitor (SERPIN) superfamily [29]. It is a single-chain glycoprotein with an apparent molecular weight of 45 kDa, consisting of 379 or 381 acid residues [30]. In humans, the PAI-1 gene is located on chromosome 7q21.3-22, it spans approximately 12 kb, and consists of nine exons [29]. The PAI-1 gene is upregulated in response to a large number of hormones, cytokines, and growth factors [29].

PAI-1 is most well known for its role in fibrinolysis—a critical hemostatic process that regulates control of clot dissolution and wound repair among other biological events. During fibrinolysis, PAI-1 acts as a fast specific inhibitor of the serine

proteases urokinase-type (uPA) and tissue-type (tPA) plasminogen activator [29,31]. Both of these plasminogen activators (uPA and tPA) are highly specific serine proteases that cleave the zymogen plasminogen, catalyzing the formation of plasmin, the primary protease essential for fibrinolysis [29, 31]. PAI-1 plays a major role in the regulation of this process; it is the primary inhibitor of the generation of the active, broad-spectrum serine protease plasmin from the zymogen plasminogen and thus, a main regulator of fibrin degradation and turnover of the extracellular matrix [29, 31]. While PAI-1 is synthesized in the active form, it has marked functional instability with a functional half-life of about 2 h in vivo. Circulating PAI-1 is bound to vitronectin, which protects the inhibitor from inactivation and may assist in targeting the inhibitor to sites of vascular injury. At least four different conformations of PAI-1 have been described: (1) the active form that reacts with plasminogen activator, (2) a latent form that is nonreactive, (3) a substrate form that can be cleaved by plasminogen activators but is noninhibitory, and (4) the inert form of PAI-1 generated by the cleavage of the reactive site. While PAI-1 is mainly produced and secreted from endothelial cells, it can also be secreted by other cell types including megakaryocytes, hepatocytes, and adipocytes [32, 33].

MCP-1, Chemerin, and PAI-1 as Adipocytokines

Adipokines, or adipocytokines, are cytokines produced in the adipose tissue. Leptin was the first adipokine to be discovered in 1994. Since then, hundreds of adipokines have been acknowledged including MCP-1, chemerin, and PAI-1. Emerging evidence identifies their importance in adipose tissue and metabolic-related diseases. Here we discuss the available literature on the impact of these novel adipokines in obesity and related pathologies.

MCP-1 and Obesity

MCP-1 gene expression and that of its receptor, CCR2, has been documented to be elevated in both the visceral fat and subcutaneous fat of obese subjects compared to nonobese controls [34]. Further, protein levels have been reported to be higher in omental fat compared to subcutaneous fat—at least for severely obese patients [35]. Similarly, experimental studies in rodents have reported increases in mRNA expression of MCP-1 and its functional receptor CCR2 in adipose tissue. For example, we have reported an increase in MCP-1 (~8-fold) in epididymal tissue following 16 weeks of consumption of a high fat diet, which was designed to be similar in content to the standard American diet [36]. Consistent with this, increased circulating levels of MCP-1 are generally found to be elevated in obese patients and mice fed a high fat diet. Although MCP-1 is largely produced by immune cells including macrophages, given its recent attention as an adipokine we now know that it is also produced

by adipocytes themselves [37]. It has been reported to be produced and secreted from human preadipocytes as well as isolated mature adipocytes [37]. MCP-1 released by adipocytes is biologically functional and can induce inflammation and activate immune cells including macrophages and CD4+ T cells [38]. Here we will discuss the available literature on the role of MCP-1 on obesity-related pathologies including inflammation, metabolic dysfunction, and adipogenesis.

The consequences of the obesity-induced increase in MCP-1 are significant given its role in macrophage recruitment and subsequent chronic inflammation—a pathophysiological mechanism that links obesity to disease risk. In fact, the chronic inflammation characteristic of obesity is largely mediated by quantitative and phenotypic changes in adipose tissue macrophages. It has been reported that approximately 45–60 % of adipose tissue cells express the F4/80 macrophage marker in obese mice, whereas only 10–15 % of cells from lean mice express this marker [39]. In addition, adipose tissue macrophages in obese mice exhibit a proinflammatory M1 phenotype, whereas those from lean mice have an anti-inflammatory M2 phenotype [40, 41]. It is well established that MCP-1 orchestrates the enrollment of monocytes into adipose tissue during obesity—a process that is necessary for adipose tissue remodeling. For example, it has been reported that MCP-1 is increased in white adipose tissue of obese subjects resulting in recruitment of bone-derived monocytes, which infiltrate the tissue from circulation [42, 43]. This finding is largely supported by mechanistic studies in animals. For instance, one study reported that MCP-1 overexpression and depletion in genetically obese and diet-induced obese mice resulted in alterations in macrophage infiltration [44]. These findings were consistent with Kamei et al. who reported an increase in macrophage accumulation following MCP-1 overexpression in adipose tissue [45]. Others have extended these findings using manipulation of the MCP-1 receptor (C-C motif chemokine receptor-2 (CCR2)) [46]. Recently, it has been reported that MCP-1 can even induce macrophage cell division in adipose tissue implants, whereas MCP-1 deficiency in vivo decreases adipose tissue macrophage proliferation [47].

Despite this convincing body of literature however, a few studies have reported no change in adipose tissue macrophage accumulation in mouse models of obesity following interruption of MCP-1 [48–50]. For example, Inouye et al. reported no change in adipose tissue macrophage number in MCP-1 deficient mice compared with wild-type mice [51]. While the inconsistencies in the literature are still largely unexplained, Galastri et al., reported that lack of MCP-1 differently affects inflammation according to the genetic background in a model of diet-induced steatohepatitis [52]. Further, we have data to show that MCP-1 deficient mice on an FVB/N background show increased adiposity and infiltration of inflammatory cells into adipose tissue and fibrosis [53]. Thus, differences in the current literature may be explained, at least in part, by strain differences in the propensities to develop metabolic diseases [54–56], which may affect disease outcome. A comprehensive study by Montgomery et al. examined strain-dependent variation in obesity-related pathologies in response to high-fat diet feeding using five common strains of inbred mice [57]. While all mouse strains examined gained a significant amount of body fat, the response for each strain differed in the level of gene expression for

MCP-1 and the macrophage marker F4/80. In addition, the duration of high-fat diet feedings, fat content of the diet, sex of the mice, housing conditions, and/or differences in gut microbiota may potentially explain the inconsistencies in the literature. Nonetheless, to date, the majority of the literature supports a role for MCP-1 on macrophage recruitment in adipose tissue.

While the role for MCP-1 in adipose tissue has largely been confined to its importance in the recruitment of macrophages, recent evidence also suggests a role for this chemokine in metabolic processes. For example, mice engineered to express an MCP-1 transgene in adipose tissue (aP2-MCP-1) are reported to be insulin resistant [45]. Insulin resistance in MCP-1 overexpression mice was confirmed by hyperinsulinemic euglycemic clamp studies showing that transgenic mice had lower rates of glucose disappearance and higher endogenous glucose production than wild-type mice [45]. Consistent with this, insulin-induced phosphorylations of Akt were significantly decreased in both skeletal muscles and livers of aP2-MCP-1 mice. MCP-1 pretreatment of isolated skeletal muscle blunted insulin-stimulated glucose uptake, which was partially restored by treatment with the MEK inhibitor U0126, suggesting that circulating MCP-1 may contribute to insulin resistance in aP2-MCP-1 mice [45]. However as with the macrophage infiltration literature, there too are inconsistencies reported for the role of MCP-1 on metabolic dysfunction. For example, Inouye et al. reported that MCP-1 deficient mice fed a high fat diet gained more weight, were glucose intolerant, had mildly increased plasma glucose, and were hyperinsulinemic compared with wild-type mice suggesting a potential beneficial effect of this chemokine on metabolism [51]. Similarly, we recently reported that MCP-1 deficient mice on an FVB/N background gained more weight and had greater metabolic dysfunction than their wild-type counterparts [53].

An increasing body of emerging evidence links adipogenesis and inflammation. If inflammation can increase adipose tissue growth, this may be the basis for a positive feedback loop in obesity. Interestingly, MCP-1 has in fact been implicated as playing a role in adipogenesis. Using a tissue engineering model for growing adipose tissue, it was reported that MCP-1 generated proportionally large quantities of new adipose tissue [58]. Similarly, a recent study reported that MCP-1-induced protein (MCP1P) induces adipogenesis in C57BL/6 mice resulting in an increase in the number of adipocytes [59]. In addition, recent reports indicate that a reduced ability to sense and respond to proinflammatory stimuli at the level of the adipocyte decreases the capacity for healthy adipose tissue expansion and remodeling, which can ultimately lead to increased HFD-induced steatosis and metabolic dysfunction [60]. Thus, MCP-1 may be a necessary component of the inflammatory response required for adipose tissue protection, remodeling, and healthy expansion.

To date, the majority of the literature supports a link between elevated MCP-1 and adiposity, macrophage accumulation, and metabolic dysfunction. However, there is a body of evidence that supports a possible necessary role for this adipokine in healthy adipose tissue functioning. It is likely that MCP-1 may play a dual role in HFD-related pathologies that is dependent on the specific model examined. Nonetheless, it is clear that MCP-1 is an important player in obesity and metabolic perturbations.

Chemerin and Obesity

Chemerin and its receptor CMKLR1 were first documented to be produced in white adipose tissue in 2007 [61, 62]. In addition, it was reported that circulating levels of chemerin were increased in obese rodent models as well as in humans. Shortly thereafter, it was reported that chemerin and its receptor are actually expressed by adipocytes themselves [61]. Both genes were found to be upregulated during the differentiation process of mesenchymal stem cells or 3T3-L1 cells into adipocytes [61]. Further, chemerin was documented to regulate mature adipocyte functions by controlling the expression of key effectors of glucose and lipid metabolism. The in vitro evidence along with follow-up investigations using rodent studies and clinical samples has led to the classification of chemerin as an adipokine. In this section, we will discuss the literature on the role of chemerin in obesity-related pathologies and subsequent metabolic diseases.

Over the last decade the link between chemerin and obesity has been well established. Circulating chemerin levels have been reported to be increased in genetic mouse models of obesity including leptin (ob/ob) and leptin receptor (db/db) deficient mice as well as diet-induced obesity models [62–65]. This is consistent with reports in humans where circulating chemerin levels have been shown to be positively correlated with body mass index and measures of central adiposity such as waist-to-hip ratio, waist circumference, and visceral adipose tissue mass [62, 66]. Further support for this relationship comes from weight loss studies where a significant decrease in chemerin levels has been reported following bariatric surgery or exercise interventions [66, 67]. It should be noted that adipose tissue is not the sole source of chemerin; it is also produced and released by the liver in addition to some immune cells and vascular cells. However, it is thought that the obesity-associated increase in chemerin is most likely secreted primarily by adipose tissue as adipose tissue transplants from obese individuals secrete significantly more chemerin than transplants from nonobese subjects [68].

In recent years, studies have focused on the exact role that chemerin and its receptor CMKLR1 may play in obesity and obesity-related pathologies. Inflammation is a well-known consequence of obesity, thus it is no surprise that chemerin contributes to this process given its documented chemoattractant properties. Studies have reported that chemerin produced by adipokines acts as a regulator for the recruitment of CMKLR1 expressing cells to adipose tissue. An association between chemerin levels and increased infiltration of macrophages into adipose tissue along with elevated expression of proinflammatory mediators including IL-6, C-reactive protein, and TNF-α has been reported [69]. Further support for this relationship comes from studies in mice deficient for chemerin and its receptor CMKLR1; mice lacking chemerin have been reported to have reduced infiltration of macrophages into adipose tissue despite no change in fat weight and mice deficient for CMKLR1 have been shown to have a reduction in the expression of proinflammatory cytokines along with a decrease in CD3+ cells [65, 70].

Along with playing a role in immune cell recruitment into adipose tissue, chemerin has also been implicated in the process of adipogenesis. Chemerin signaling has been reported to be essential during the early clonal expansion phase of adipocyte differentiation; it is thought that peroxisome proliferator-activated receptor gamma, the master regulator of adipogenesis, increases the expression of chemerin [71]. Further evidence for this association comes from manipulation studies wherein reduction of chemerin or CMKLR1 in vitro, through the use of knockout down or neutralization, results in impairment of both 3T3-L1 and murine mesenchymal stem cell differentiation into mature adipocytes [61, 71, 72]. Further, in vivo studies have reported that CMKLR1 knockout mice have reduced body mass and adiposity in response to high-fat diet feedings. These mice have been reported to eat less food, which suggests that chemerin may play a role in the regulation of energy balance [65]. Chemerin has also been implicated in promoting angiogenesis. Thus, it is possible that chemerin may promote adipogenesis through angiogenic properties as increased angiogenesis is necessary to support expanding adipose tissue [73].

Based on the current literature, it is clear that the adipokine chemerin plays a role in obesity-related pathologies. Thus, it is not surprising that chemerin has been implicated in a variety of obesity-related diseases including type 2 diabetes and metabolic syndrome. The available evidence implicates an effect of chemerin and its receptor CMKLR1 on glucose homeostasis and type 2 diabetes. Administration of exogenous chemerin exacerbates glucose tolerance, lowers serum insulin, and decreases tissue glucose update in obese/diabetic mice but not normal glycemic mice implicating a role for this adipokine in the metabolic derangements associated with type 2 diabetes [64]. A follow-up study by the same group reported that CMKLR1 knockout mice fed a diet high in fat were glucose intolerant and exhibited decreased glucose stimulated insulin secretion as well as decreased skeletal muscle and white adipose tissue glucose uptake indicating a role for CMKLR1 in glucose homeostasis [65]. In addition, chemerin deficient mice have been reported to be glucose intolerant—an effect that has been linked to increased hepatic glucose production and impaired insulin secretion as impaired glucose-dependent insulin secretion was reported in chemerin deficient mice following studies using isolated islets and perfused pancreas whereas chemerin overexpression in mice revealed enhanced glucose-dependent insulin secretion and improved glucose tolerance [70]. Using recombinant adeno-associated virus to express human chemerin in LDL receptor knockout mice that were exposed to high-fat diet feedings, it was reported that chemerin significantly increased glucose levels during a glucose tolerance test without affecting endogenous insulin levels and the insulin tolerance test [74]. Although it is clear from the aforementioned mouse studies that chemerin and its receptor, CMKLR1, play a role in regulating glucose homeostasis, there are inconsistencies in the literature with regards to the exact role that these factors play in this process; it appears that depletion of chemerin as well as its overexpression result in impaired glucose tolerance. Human studies have also indicated a potential role for chemerin in type 2 diabetes and glucose homeostasis as elevated chemerin has been reported in the serum and adipose tissue of type 2 diabetic patients [66, 67]. Further, serum chemerin concentration is strongly linked to insulin sensitivity in type 2 diabetic

individuals [75]. The role for chemerin in glucose tolerance and development of type 2 diabetes is undoubtedly complex. Although mechanistic studies in animals have advanced our understanding of the role of this adipokine, many questions still remain. Based on the available literature it appears that normal chemerin signaling is necessary for proper glucose regulation.

Chemerin has also been implicated in the development of metabolic syndrome; it has been reported that elevated circulating chemerin is a significant risk factor for the metabolic syndrome [76]. Moreover, levels of serum and adipose tissue derived chemerin are elevated in patients with metabolic syndrome and have been reported to be positively correlated with circulating triglycerides, low-density lipoprotein cholesterol, waist circumference, insulin resistance, fasting insulin/glucose, and systolic as well as diastolic blood pressure—characteristics of the metabolic syndrome [62, 76–80]. Many of these alterations have been reported to be reversed following exercise-induced weight loss and subsequent decrease in chemerin including insulin resistance, fasting blood glucose, fat mass, and waist circumference [81].

To date, the majority of the literature supports a link between elevated chemerin and obesity that is consistent with an abundance of metabolic and inflammatory perturbations. However, whether chemerin is playing a pathogenic or protective role in obesity-related pathologies is still unclear but is likely to be dependent on the physiological context. Chemerin activity appears necessary for normal healthy adipose and immune function; it is likely that the dysregulation of chemerin resulting in either abnormally increased or decreased expression contributes to the development of pathological processes.

Plasminogen Activator Inhibitor 1 and Obesity

Clinical studies have clearly demonstrated that obesity is associated with impaired fibrinolysis. Thus, it is no surprise that PAI-1 has been implicated in obesity-related pathologies. As adiposity increases, so too does the production of PAI-1 leading to an impairment of the fibrinolytic system. PAI-1 is synthesized and secreted by adipose tissue; Skurk and Hauner reported that the greater the fat cell size and the adipose tissue mass, the greater is the contribution of adipose production to PAI-1 [82]. It has been correlated with a number of obesity-related outcomes including body mass index, waist circumference, waist-to-hip ratio, total fat mass, visceral adipose tissue, subcutaneous adipose tissue, HOMA index, inflammatory biomarkers, insulin, glucose, triglycerides, and cholesterol. In this section, we will examine the available literature linking obesity to PAI-1.

PAI-1 was first reported to be expressed in adipose tissue of untreated mice in 1991 [83]. This response appeared to be regulated by inflammatory mediators as treatment of mice with LPS, TNF-α, or TGF-β increased mRNA expression of PAI-1 in adipose tissue [83]. In a subsequent study by the same group, it was reported that genetically obese (ob/ob) mice have a fivefold higher concentration of circulating PAI-1 than their lean counterparts [84]. Similarly, PAI-1 expression was

significantly elevated in the epididymal, subcutaneous, and brown adipose tissue of obese mice compared to lean control mice [84]. However, it should be noted that visceral adipose tissue has been reported to have a greater capacity to produce PAI-1 than subcutaneous adipose tissue [82]. Although PAI-1 expression was reported to be increased in other major organs with an obesity phenotype, the magnitude of increase was much greater in the adipose tissue depots. This is not surprising given that the size and number of adipocytes, and thus, the amount of adipose tissue mass, increases several fold in obesity. Therefore, as adiposity increases it is likely to enhance the biosynthetic capacity of PAI-1. Further evidence for this relationship comes from in situ hybridization studies of adipose tissue from obese mice in which PAI-1 mRNA is increased in adipocytes [85]. Similarly, in vitro studies have demonstrated that mature, fully differentiated 3T3-L1 adipocytes in culture produce elevated levels of PAI-1 mRNA and protein [85]. It is thought that the obesity-induced induction of PAI-1 occurs, at least in part, via proinflammatory cytokines including IL-6 and TNF-α, which have been well documented to increase with an obesity phenotype [86–88]. However, it has also been suggested that PAI-1 synthesis can be upregulated by insulin, glucocorticoids, and angiotensin II as well as some fatty acids [82].

While it is clear that increased PAI-1 is correlated with obesity, studies using knockout mice have been performed in order to further our understanding of the role of this adipokine in obesity-related pathologies. It has been reported that PAI-1 deficient mice are protected against insulin resistance and development of obesity following feedings with a high-fat/high-carbohydrate diet [89]. Further, PAI-1 deficient mice have increased metabolic rates and total energy expenditure compared to wild-type mice [89]. In addition, insulin sensitivity is enhanced as is the expression of key molecules involved in insulin sensitivity including PPAR-gamma and adiponectin following ablation of PAI-1 [89]. Similarly, treatment of wild-type mice with angiotensin type 1 receptor antagonist to downregulate PAI-1 inhibited PAI-1 increases and ameliorated diet-induced obesity, hyperglycemia, and hyperinsulinemia [89]. PAI-1 deficiency also enhanced basal and insulin-stimulated glucose uptake in adipose cells in vitro [89]. These data suggest that PAI-1 may not merely increase in response to obesity and insulin resistance but it may in fact have a direct causal role in the development of obesity and insulin resistance [89].

Consistent with the animal literature, clinical studies also support a relationship between PAI-1 and obesity [90]. Expression of PAI-1 has been documented in the adipose tissue of human subjects. Further, omental tissue explants produced significantly more PAI-1 antigen than subcutaneous tissues from the same individual. Moreover, clinical studies demonstrated that weight loss due to surgical treatment, dietary intervention, or exercise significantly reduced plasma PAI-1 levels in obese humans indicating that the obesity-induced increase in PAI-1 is reversible [91, 92]. To further examine the role of PAI-1 in the development of human adiposity, Hoffstedt et al. examined whether the PAI-1 gene may cause obesity [93]. They investigated the frequency of a −675 4G/5G promoter polymorphism in the PAI-1 gene in 188 lean, 70 overweight, and 247 obese, but otherwise healthy Scandinavian subjects [93]. A deletion/insertion polymorphism within the PAI-1locus (4G/5G) affected the expression

of this gene [93]. The deletion of 4G was associated with significantly higher concentrations of PAI-1 than the 4G/5G insertion [93]. Concentrations of PAI-1 in homozygous 4G individuals were reported to be 25 % higher than that observed in 5G homozygotes [93]. Interestingly, homozygosity for 4G was more common among obese individuals, whereas homozygosity for 5G occurred more frequently in lean subjects [93]. The study concluded that the −675 4G/5G polymorphism in the PAI-1 gene is strongly linked to obesity in the 4G homozygous form [93].

The mechanisms responsible for the PAI-1 induced increase in body weight and related perturbations have yet to be established. It has been suggested that PAI-1 may affect fat tissue growth by changing receptor-dependent transport of lipids into the lipocytes [94]. Alternatively, PAI-1 inhibition may block angiogenesis that would ultimately weaken the vascularization and consequent growth of the adipose tissue [94]. Finally, it has been postulated that inactivation of PAI-1 may stimulate migration of preadipocytes that would prevent their full differentiation into mature fat cells [94].

Given the link between obesity and type 2 diabetes, it is no surprise that PAI-1 is elevated in type 2 diabetes patients. A 5-year study by Festa et al. that included a total of 843 individuals reported that PAI-1 levels were related to the incidence of diabetes [95]. Progression of PAI-1 levels over time was associated with rising glucose levels and the development of type 2 diabetes [95]. Further, PAI-1 has been linked to diabetic nephropathy and diabetic vascular disease, both of which lead to poor prognosis for the patient.

The literature to date clearly implicates an association between elevated PAI-1 and obesity. What is less clear is a complete understanding of the mechanisms by which PAI-1 contributes to obesity. Future development of therapeutic strategies to inhibit PAI-1 will rely on characterization of the exact mechanisms linking PAI-1 to obesity.

MCP-1, Chemerin, and PAI-1 in Cancer

Although MCP-1, chemerin, and PAI-1 have clearly been implicated in the development of obesity and associated pathological conditions, they are probably equally known for their roles in cancer development. This is not surprising given that receptors for each of these cytokines are present on immune cells, which play a critical role in the cancer progression. Here we discuss the available literature on the impact of these adipocytokines in the development of tumorigenesis.

MCP-1 and Tumorigenesis

Elevations in MCP-1 have been implicated in the development and progression of tumorigenesis; there is an accumulating body of literature that supports a role for MCP-1 in the development of breast cancer and colon cancer, among others. The mechanisms linking MCP-1 to carcinogenesis have largely been attributed to its

ability to recruit macrophages to the tumor microenvironment. Macrophages are major players in the connection between inflammation and cancer [96, 97]; they have been reported to represent up to 50 % of the tumor mass and have the ability to produce a wide array of inflammatory mediators with protumoral functions [96, 97]. In general, high levels of tumor-associated macrophages have been associated with poor prognosis in various cancers [96–100]. Thus, targeting MCP-1 may be a viable therapeutic strategy to reduce macrophage infiltration to the tumor microenvironment. Here we will discuss the evidence linking MCP-1 to tumorigenesis.

A role for MCP-1 in tumorigenesis is documented in the clinical literature [101]. Perhaps the most convincing evidence comes from studies examining the relationship between MCP-1 and mammary tumorigenesis. The available evidence suggests a strong tumorigenic role for MCP-1 in mammary cancer through its effects on monocyte/macrophage recruitment and activation, induction of angiogenesis, as well as promotion of metastasis [102–104]. Disease-free breast epithelial cells lack expression of MCP-1, while expression is significantly elevated in both neoplastic and stromal cells within the breast tumor microenvironment [103–110]. The expression of MCP-1 appears to be an acquired feature gained during tumor development implicating it as advantageous to the establishment and development of the tumor. A number of studies have reported that MCP-1 is significantly correlated with high tumor grade and lymph node metastasis as well as being associated with low levels of differentiation and poor prognosis [103, 111–113]. Expression of MCP-1 has been characterized in both noninvasive ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC), while being negligible in the adjacent normal breast epithelial cells of both of these conditions [103, 105–108]. However, the magnitude of increase in MCP-1 is varied; human samples of IDC exhibit a wide range of MCP-1 expression with levels reported to vary between 17 % and ~90 % positivity within tumor or stromal cell populations [103, 105, 106, 108, 109]. Of the stromal cell types, macrophages and fibroblasts have been reported to exhibit the highest incidence of MCP-1 expression [103, 108]. Given that several cell types within the tumor microenvironment express and respond to MCP-1, it exhibits both autocrine and paracrine growth promoting effects. Colon cancer studies have also implicated a role for MCP-1 on tumorigenesis. For example, Bailey et al. reported that the expression of MCP-1 increased in solid cancers with tumor stage and MCP-1 production was localized to tumor cells [101].

Consistent with the clinical literature, rodent studies have associated MCP-1 levels with tumorigenesis [101, 114, 115]. A relatively recent report from our laboratory shows that MCP-1 gene expression is correlated with the number of large polyps in the $Apc^{Min/+}$ mice mouse model of intestinal tumorigenesis [116]. In order to further explore the relationship between MCP-1 and colon cancer, we introduced MCP-1 deficiency into the $Apc^{Min/+}$ mouse and examined intestinal tumorigenesis [117]. We found a reduction in overall intestinal polyp number (~20 %) as well as the number of large polyps (~45 %), which was consistent with an increase in apoptosis [117]. Based on these data we concluded that MCP-1 can affect both the development and the growth of polyps. However, it appears as if it plays a larger role in progression of growth as opposed to initiation of development given that greater

magnitude of change in polyp growth with MCP-1 deficiency. Consistent with our findings, another rodent study shows a decrease in tumor size in a mouse model of colitis-associated colon cancer that is deficient for the MCP-1 receptor [115]. Further, it has been reported that liver metastasis following intrasplenic inoculation of MC38 colon carcinoma cells is dependent, at least in part, on MCP-1 as inhibition of MCP-1 signaling and absence of its cognate receptor CCR2 decreased tumor burden [118]. Using the anti-inflammatory molecule bindarit to target MCP-1, we investigated the role of this chemokine on mammary tumorigenesis in the C3(1)/SV40Tag transgenic mouse model of breast cancer [119]. Bindarit treatment (MCP-1 inhibition) reduced tumor number, but did not affect tumor size, tumor weight, or tumor latency in C3(1)/SV40Tag mice [119]. Within the tumor, mRNA expression of bindarit's primary targets, MCP-1 and IL-12/p35, was significantly decreased by bindarit treatment [119].

It is thought that the primary means by which MCP-1 exerts its protumor effects is as a powerful monocyte and macrophage chemoattractant. In a mouse model of intestinal tumorigenesis, we reported that MCP-1 deficient $Apc^{Min/+}$ mice have decreased macrophage number in both the polyps and surrounding intestinal tissue [117]. Specifically, we found that MCP-1 deficiency reduces the expression of both M1 (IL-12 and IL-23) and M2 (IL-13, CD206, TGF-β, and CCL17) macrophage phenotypic markers in the tumor environment as well as surrounding intestinal tissue in the $Apc^{Min/+}$ mouse model of colon cancer—this was associated with a decrease in select inflammatory mediators in the intestinal tissue as well as in circulation [117]. Similarly, a reduction in macrophage infiltration, that was consistent with a decrease in the size of colon polyps, has been reported in an MCP-1 receptor deficient mouse model of colitis-associated colon cancer [115]. Analysis of the macrophage infiltrate in human colon tissue showed that accumulation was significantly greater in tumors than controls and within tumors it was the greatest in necrotic regions [101]. Macrophage accumulation was found to be increased with tumor stage and it correlated with MCP-1 expression [101]. Further, macrophages in the tumor microenvironment are associated with MCP-1 expression in mammary tumors and have been reported to promote several tumorigenic actions including the expression of growth enhancers, angiogenic factors, and mediators of inflammatory processes [103, 104, 120, 121]. Incidentally, tumor-associated macrophages themselves are also a potent source of MCP-1, leading to continued macrophage recruitment and tumor enhancement [104]. This has been documented in vitro by coculture of macrophages with MDA-MB-231 breast cancer cells, which resulted in significantly increased MCP-1 expression and chemotactic activity of the macrophages, as well as in vivo where anti-MCP-1 treatment of mice bearing MDA-MB-231 mammary tumors decreased macrophage number in association with tumor volume [104]. Additionally, endogenous anti-MCP-1 treatment has been shown to inhibit primary tumor growth in mice, most likely due to a decrease in macrophage recruitment to the tumor microenvironment [104, 122]. In fact, we have reported that C3(1)/SV40Tag transgenic mammary cancer mice treated with an MCP-1 inhibitor have a reduction in the expression of select macrophage markers in the mammary tissue, which was associated with a decrease in tumor number

[119]. Human IDC breast cancer samples have provided further evidence for the link between tumor-associated macrophages and MCP-1 expression in breast cancer. Within the stroma, tumor-associated macrophages are thought to be the predominant MCP-1 expressing cell type [105, 108] and stromal expression of MCP-1 has been associated with macrophage accumulation [103–105]. High levels of macrophages in the tumor microenvironment reported in IDC are indicative of poor prognosis and disease progression and have been associated with lymph node involvement [104, 105, 113]. However, literature supporting no association between MCP-1 and macrophages evaluated in the stroma and tumor cells of human IDC in situ also exist [104, 109]. Furthermore, variability exists between cancer location as infiltrative lobular carcinoma samples provide no evidence of a link between macrophage accumulation and other predictive variables including tumor size, metastatic lymph node involvement, stage of cancer, microvascular density (MVD), vascular endothelial growth factor (VEGF), tumor grade, or mitotic activity index [123]. The lack of consistent findings among available studies may be due to the heterogeneous nature of the disease. Future investigations are necessary to fully understand the importance of MCP-1 in breast cancer and its potential as a prognostic indicator and/or therapeutic target.

Chemerin and Tumorigenesis

Emerging evidence links chemerin to the development and progression of cancer, although the literature is still relatively sparse—especially on the preclinical side. Nonetheless, several studies have shown that the expression of chemerin is dysregulated in several types of tumors. A number of clinical studies have reported that chemerin expression is associated with protumorigenic outcomes [124–127]. Interestingly however, chemerin expression is downregulated in some cancers including melanoma, skin squamous cell carcinoma, lung carcinoma, and hepatocellular carcinoma, which has been associated with poor differentiation [128–130]. In this section, we will discuss the available literature linking chemerin to tumorigenesis.

Chemerin has been implicated as playing a role in a variety of cancer models including colon cancer, gastric cancer, and squamous cell carcinoma of the oral tongue cancer. A recent clinical study examined chemerin in colon cancer patients versus controls and found a significant increase in circulating chemerin implicating a link between chemerin and risk of developing colorectal cancer [124]. Similarly, the relationship between preoperative plasma chemerin levels and prognosis of gastric cancers was evaluated [125]. Plasma chemerin levels were significantly elevated in gastric cancer patients compared to controls and it was identified as an independent predictor for 5-year mortality, overall survival, and disease-free survival [125]. Further evidence for a role of chemerin in gastric cancer was reported by Wang et al., who sought to determine the serum levels of chemerin in gastric cancer patients and healthy subjects and to investigate the biological effect of chemerin on

gastric cancer cells [126]. They found that chemerin levels were significantly higher in gastric cancer patients compared to controls and was associated with advanced clinical states [126]. Further, it was reported that chemerin increased invasiveness of gastric cancer cells [126]. Chemerin overexpression has also been associated with tumor angiogenesis and poor clinical outcomes of squamous cell carcinoma of the oral tongue implicating that chemerin could be a new therapeutic target for regulating tumor angiogenesis and blocking tumor progression [127]. This adipokine has also been implicated as playing a role in altering mesenchymal stem cell function; chemerin increased adhesion of mesenchymal stem cells and adhesion, proliferation, and migration of myofibroblasts [131].

However, not all of the literature points to a positive correlation between chemerin levels and tumorigenesis. Some studies indicate no relationship between chemerin and carcinogenesis while some studies in fact report a negative relationship where low levels of chemerin are associated with poor prognosis. Imai et al. examined the relationship between circulating chemerin and hepatocellular carcinoma and reported no significant correlations between serum chemerin levels and recurrence-free survival or overall survival in hepatocellular carcinoma patients [130]. In the same cancer model, it was reported that chemerin expression was significantly decreased in patients with hepatocellular carcinoma and survival analysis revealed that patients with lower chemerin expression and poorer survival than those with higher expression [129]. This was linked to a lowered infiltration of both dendritic cells and natural killer cells suggesting an immune-mediated mechanism [129]. Using whole genome expression datasets it was reported that the chemerin gene is downregulated in melanoma as well as other human tumors [128]. Further, high chemerin mRNA expression in tumors was associated with improved outcome in human melanoma [128]. This finding was further validated using the B16 transplantable mouse melanoma model where tumor-expressed chemerin inhibited in vivo tumor growth [128]. Further, intratumoral injection of chemerin inhibited growth suggesting the potential for therapeutic application [128]. The mechanism responsible for this effect is thought to be due to an alteration in the tumor-infiltrating cells as an increase in natural killer (NK) cells and a relative reduction in myeloid-derived suppressor cells and putative immune inhibitory plasmacytoid dendritic cells was associated with growth inhibition [128]. Thus, chemerin may be necessary for adequate immune activation and prevention of immune suppression in the tumor microenvironment [128].

Based on the available literature it is not completely clear whether or not chemerin exerts protumor or antitumor effects. This is hampered by the fact that the majority of the published literature is reporting on clinical studies with preclinical studies using mice virtually nonexistent. Some of the available literature suggests that chemerin may promote tumorigenesis whereas others indicate a benefit of chemerin expression on suppressing tumorigenesis suggesting that it may be model specific or perhaps even dependent on the stage of the cancer. Extensive studies and especially preclinical studies are necessary to establish firm conclusions on the role of chemerin on tumorigenesis and associated mechanisms.

Plasminogen Activator Inhibitor 1 and Tumorigenesis

PAI-1 is reported to be expressed in many cancer cell types and is regarded as a potential biochemical marker for poor prognosis [132–134]. Studies have demonstrated that PAI-1 is a potent regulator of tumor growth in vivo and increased PAI-1 has been confirmed in many solid tumor types and found to be associated with poor outcomes [132–135]. Therefore, PAI-1 may serve as an important therapeutic target for certain cancers. In fact, PAI-1 inhibitors are now being tested, at least in preclinical models of cancer. Here we will discuss the recent evidence linking the novel adipokine PAI-1 to tumorigenesis.

There is substantial clinical evidence that has linked PAI-1 to tumorigenesis. Several retrospective and prospective studies have shown that elevated levels of PAI-1 in breast tumor tissue are statistically independent and potent predictors of poor patient outcome, including adverse outcome in the subsets of breast cancer patients with lymph node-negative disease [132–134]. Similarly, PAI-1 levels of epithelial ovarian cancer patients were associated with cancer stage, residual tumor size, and lymph node metastasis [136]. Likewise, it has been documented that lower expression of PAI-1 in the tumor cells of oral squamous cell carcinoma is associated with low disease specific death in patients with small tumors and no lymph node metastasis [135]. A recent study reported that circulating PAI-1 levels were higher in colorectal cancer patients with liver metastasis and correlated with liver metastasis, tumor size, differentiation, serosa infiltration, Duke's stage, and lymphatic metastasis [137]. In addition, PAI-1 protein levels in the colorectal cancer tissue of patients with liver metastasis were significantly greater than that in those without liver metastasis [137]. To further confirm the role of PAI-1 in this model, colorectal cancer cells were transfected with lentivirus expression PAI-1 small interfering RNA and it was reported that the proliferation, invasion, and migration abilities of these cells were significantly reduced [137]. Similarly, PAI-1 has been associated with high-grade dysplasia and carcinoma suggesting a functional role for PAI-1 in malignant transformation in colitis-associated colon cancer [138]. Taken together, the clinical literature provides strong support for the development of PAI-1 as a diagnostic marker to identify patients at risk for cancer.

Animal studies have provided strong evidence to support a role for PAI-1 on tumorigenesis. Convincing evidence comes from studies using PAI-1 deficient murine tumor cells syngeneically implanted into wild-type or PAI-1 deficient mice documenting the contributory role of host and tumor-derived PAI-1 to tumor growth, angiogenesis, and metastasis [137, 139–143]. For example, nude mice inoculated with PAI-1 knockdown colon cancer cell lines had fewer metastatic nodules in the liver and smaller tumor volumes [137]. The greatest antitumor effects were shown when PAI-1 was suppressed in both the host and the tumor cells. Similar findings were reported when PAI-1 deficient human tumor cells were xenotransplanted in immunodeficient PAI-1 null mice [144–147]. In general, the findings imply that deficiency of PAI-1 expression in human tumor cells and in mouse host cells resulted

in slower tumor growth. In contrast however, investigations using genetically engineered mouse models of cancer crossed with PAI-1 deficient mice do not show any effect on tumor initiation, growth, or metastasis following PAI-1 suppression. For example, deletion of PAI-1 had no effect on tumorigenesis in an Apc/Apc1638N mouse model of colon cancer [148]. Similarly, there was no effect of PAI-1 ablation on mammary tumors, metastasis, or survival in a PyMT mouse model of breast cancer [149]. One possible explanation for the disparate findings between transplanted and genetically engineered mice is the potential presence of compensatory serpins including PAI-2, protein C inhibitor, or maspin, whose overexpression may compensate for a lack of PAI-1 [150].

Several mechanisms support the protumorigenic activity of PAI-1. PAI-1 has been reported to have angiogenic activity, which is crucial to tumor viability and dissemination. However, this phenomenon is thought to be dose dependent as physiological concentrations of PAI-1 have been documented to promote angiogenesis whereas pharmacological concentrations inhibit angiogenesis [151]. The precise mechanism whereby PAI-1 promotes angiogenesis has not yet been fully elucidated but it is thought to be dependent on its protease inhibitor activity, not vitronectin binding [139]. In addition, PAI-1 has the ability to inhibit apoptosis in cancer cells. It has been reported to inhibit Fas-mediated apoptosis in a number of human cancer cell lines through its control over pericellular plasmin activity [145, 152]. Further, PAI-1 has been documented to affect intrinsic apoptosis as the absence of extracellular PAI-1 in tumor cells results in higher levels of activated caspase-9 [153]. Finally, intracellular PAI-1 promotes cell survival through its ability to inhibit caspase-3, which protects tumor cells from chemotherapy-induced apoptosis [154].

Given the role of PAI-1 in cardiovascular disease, a variety of small molecule PAI-1 inhibitors have been developed. While their efficacy in cancer models is limited, a number of preclinical studies have reported promising results for the use of PAI-1 inhibitors on preventing tumorigenesis. For example, SK-216 orally administered to Apc/Apc1638N mice for the duration of 9 weeks resulted in almost a two-fold reduction in intestinal polyps [155]. Similarly, SK-216 administration decreased tumor size and inhibited metastases and angiogenesis in a mouse model of Lewis lung carcinoma [156]. Consistently, administration of the PAI-1 inhibitor PAI-039 to mice xenotransplanted with human T24 bladder and HeLa cervical cancer cells resulted in significant reduction in tumor volume, which was associated with a decrease in tumor cell proliferation and vascularization and an increase in apoptosis [157]. These preclinical studies provide strong support for the further investigation and development of PAI-1 inhibitors in cancer models.

In summary, the clinical and preclinical literature clearly supports a role for PAI-1 in the advancement of tumorigenesis making it a potentially useful biomarker for the assessment of cancer progression. The mechanisms linking PAI-1 to tumorigenesis are likely to occur via its documented effects on promotion of angiogenesis and inhibition of apoptosis making it a viable cancer therapy target. In fact, PAI-1 inhibitors have shown promise on slowing tumor growth and preventing metastasis. However, the short half-life, the low bioavailability, the lack of activity of these inhibitors on some forms of PAI-1, and the potential systemic effect that chronic

administration of PAI-1 inhibitors may have on hemostasis provide challenges to limit use of the current forms of PAI-1 inhibitors for cancer therapy. New therapies with improved pharmacological profiles may provide an efficacious cancer therapy in the clinic.

Conclusions

The novel adipokines MCP-1, chemerin, and PAI-1 are clearly linked to obesity and cancer. The majority of evidence indicates that it is the overexpression of these factors that drives obesity and tumorigenesis. In general, MCP-1, chemerin, and PAI-1 have been reported to be increased in models of obesity and cancer and are associated with worsened outcomes. However, there is also literature to suggest that depletion or knockdown of these adipokines may drive disease states—at least in some models. Thus, whether these adipokines are playing a pathogenic or protective role is likely to be dependent on the physiological context.

While there is a reasonable body of evidence linking MCP-1, chemerin, and PAI-1 to obesity and cancer, literature on the role of these adipokines in driving obesity-enhanced tumorigenesis is simply not yet available. However, given that the majority of literature implicates that these adipokines to be increased in the independent diseases, it is almost a certainty that these factors play a role in obesity-enhanced cancer. This has certainly been the case for the more well-known adipokines like leptin and adiponectin wherein studies have implicated a positive association between leptin and obesity-enhanced tumorigenesis and a negative association for adiponectin. As such, future studies using models of obesity and tumorigenesis will be required to establish a role for MCP-1, chemerin, and PAI-1 in driving obesity-enhanced cancer.

As these adipokines have clear roles in processes independent of obesity and cancer, inhibitors for these novel adipokines have already been developed and used at least in the preclinical literature. In the case of PAI-1 and MCP-1, these inhibitors have already been tested in animal models of tumorigenesis with some success. Thus, continued research to establish whether these novel adipokines may present a potential new therapeutic target for obesity and cancer is necessary.

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Chapter 9
Resistin, Obesity, and Cancer

Zhenzhen Zhang, Jackilen Shannon, and Hanrui Zhang

Abstract Obesity, a major global epidemic of this century, is associated with type 2 diabetes, cardiovascular diseases, and various types of cancer. Cancer is among the leading causes of morbidity and mortality worldwide. Many adipose tissue-derived biomarkers are associated with risks of cancer. Resistin is an adipokine produced mainly by adipocytes in mice, but monocytes and macrophages in human. Resistin was initially recognized as a regulator of insulin resistance and inflammation. There has been increasing evidence suggesting an association between increased resistin levels and obesity, as well as obesity-related disorders, such as diabetes, cardiovascular diseases, and malignancies. In particular, studies have reported higher circulating resistin levels in patients with certain types of cancer including breast cancer, colorectal cancer, lung cancer, hematologic malignancies, endometrial cancer, and pancreatic cancer, but contradictory results have also been documented. Although our understandings of the causal role and the underlying mechanisms of resistin in cancer progression and metastasis remain incomplete, resistin has been found to promote proliferation, migration, and invasiveness of a number of cancer cell lines. Furthermore, as a key inflammatory biomarker and mediator of insulin resistance, resistin may indirectly mediate cancer development through its metabolic effects. In this chapter, we summarize the current findings on the pathophysiological implications of resistin in obesity and related diseases from experimental, clinical, and epidemiological perspectives in order to decipher its role in linking obesity, insulin resistance, and inflammation to cancer.

Keywords Resistin • Obesity • Diabetes • Cancer • Inflammation • Insulin Resistance • Biomarker

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Introduction

Obesity has become a global health problem over the past decades. Recent statistics showed that the prevalence of overweight [body mass index (BMI) ≥ 25 kg/m^2] was 39 % and the prevalence of obesity (BMI ≥ 30 kg/m^2) was 13 % among adults aged ≥ 18 years worldwide in 2014 [1]. Obesity results from energy imbalance between energy intake and expenditure [2] and increased food energy supply was the driving force behind the current obesity epidemic [3]. Obesity is a well-established risk factor for multiple chronic diseases including type 2 diabetes, cardiovascular diseases [4–7] and several forms of cancer [8]. It was estimated that 3.6 % of all new cancer cases in adults aged 30 years and older were attributable to high BMI in 2012 [9]. However, the biological mechanisms underlying theses associations warrant further investigation.

Adipose tissue serves as an endocrine organ that secretes bioactive substances known as adipokines [10–12]. Laboratory and epidemiological studies suggest that, in obesity, the dysregulation of adipokine produced by adipose tissue can contribute to the diverse consequences of obesity including diabetes, cardiovascular diseases, and cancers, which all share common pathways involving insulin resistance and chronic inflammation [10–12]. Resistin, with its name coming from "resistance to insulin," is one of the adipokines representing an important link between obesity, inflammation, and insulin resistance [4–7], and has been implicated to be associated with increased risk of cancer. Resistin has raised considerable research interest since it was first discovered. Figure 9.1 illustrates the number of articles on resistin as indexed in PubMed by year. A number of review articles have summarized resistin's biochemical and biological roles in obesity, obesity-associated metabolic disorders [4–7], and malignancies [13–15]. This chapter reviews the pathophysiological role of resistin, the controversies regarding the association between resistin and cancer risk, and the mechanisms underlying the potential role of resistin in carcinogenesis based on a growing body of evidence from recent biomedical, clinical, and epidemiological studies.

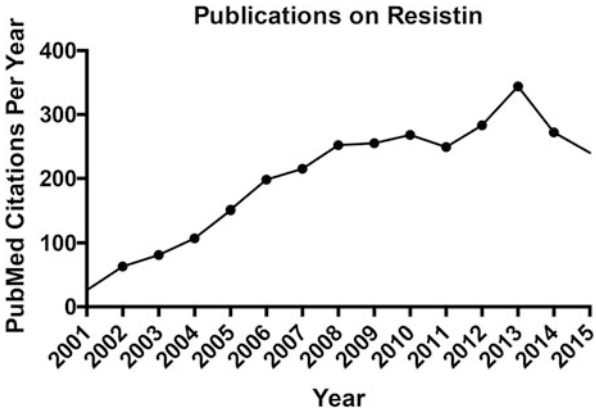

Fig. 9.1 The *graph* shows the number of papers published since 2001, the year when resistin was first identified, in PubMed using "resistin" as the search term

Resistin Biology

Resistin (*Retn*) was first identified in 2001 as a gene abundantly expressed and secreted by murine adipocytes [16]. In contrast to rodents, resistin is mainly expressed in monocytes and macrophages of the stromal-vascular fraction of adipose tissue in human [17]. As discussed below, laboratory and epidemiological studies suggested the role of resistin in obesity and type 2 diabetes, which are related to an increased risk of cancer [18].

Identification and History of Discovery

Resistin belongs to a family of cysteine-rich secretory proteins called resistin-like molecules (RELM) [16, 19] or FIZZ (found in inflammatory zones) proteins [20]. This family of proteins is characterized by a unique spacing of 10–11 cysteine residues. Three research teams discovered resistin independently [5]. Steppan et al. identified resistin in a screen for genes that were induced during 3T3-L1 adipocyte differentiation but downregulated in mature adipocytes exposed to rosiglitazone, an anti-diabetic drug in the thiazolidinedione (TZD) class [16]. Kim et al. identified resistin as an adipose secretory factor by microarray analysis of murine white adipose tissue (WAT) [21]. Holcomb et al. found resistin, which they termed '*FIZZ3*', as a member of a gene family whose founding member, *FIZZ1*, was implicated to be induced during lung inflammation [20].

Resistin Gene Expression and Secretion

Human resistin pre-peptide is 108 amino acids (aa) in length, and contains an 18 aa signal sequence plus a 90 aa mature region. The murine resistin mRNA encodes a 114 aa polypeptide containing a 20 aa signal sequence [6, 13]. Human and rodent resistin share about 60 % sequence homology [20, 22] and are syntenic. The mouse gene encoding resistin is located on mouse chromosome 8A1 and human gene encoding resistin is located on chromosome 19p13.3 [6].

In rodents, resistin is primarily expressed in mature white adipocytes. The adipocyte specificity of resistin gene expression in rodents is because of a novel adipocyte-specific enhancer element upstream to the transcription start site (TSS) of *Retn*, which contains binding sites for peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer-binding protein-alpha (C/EBP-α) [23, 24]. Resistin is secreted into the medium by differentiated 3 T3-H1 adipocytes and is detectable in mouse serum [16]. Resistin circulates in two distinct forms in mice: the major form is a hexamer composing of two trimers that are linked by intermolecular disulfide bonds, and a minor form as smaller trimeric proteins assembled through coiled-coil

interactions by resistin protomer. The trimer migrates as a monomer on non-reducing gels, indicating the absence of disulfide bonds involved in hexamer stabilization [25]. A single cysteine at position 26 has been suggested to be responsible for the covalent oligomerization of resistin because mutation of this cysteine to alanine (C26A) produces a molecule that is unable to form the disulfide bond to produce the hexamer. Infusion of the C26A mutant resistin revealed more potent effects on ameliorating hepatic insulin sensitivity in pancreatic insulin clamp studies [26]. However, monomeric C26A resistin, lacking the intertrimer disulfide bonds, had no inhibitory action on glucose uptake in mouse cardiomyocytes [26].

Unlike rodents, human resistin is mainly expressed in monocytes and macrophages, and its expression in human adipose tissue is predominantly due to non-adipocyte resident inflammatory cells [27–30]. The lack of human resistin expression in adipocytes appears to be attributable to the loss of a genomic binding site for the nuclear receptor PPAR-γ, which controls the adipocyte-specific expression of the mouse *Retn* gene [24]. Visceral adipose tissue is the primary source of resistin production compared to other adipose tissue depots. It is estimated that resistin production was 2.5-fold greater from human abdominal omental adipose tissue than subcutaneous adipose tissue [30]. In human serum, there are also different molecular isoforms of circulating resistin such as trimeric and oligomeric forms, and the oligomeric form is considered to have a more potent effect on the stimulation of proinflammatory cytokines [31, 32]. Thus, structural transition of resistin, whether concentration-dependent or not, may be involved in maintaining the fine balance in the regulation of resistin function.

Resistin Receptors and Resistin Signaling Pathways

As a secreted, circulating polypeptide, resistin's effects may be mediated through both endocrine and paracrine modes, likely through receptors on the surface of target cells. Previous reports have suggested an isoform of decorin [33], mouse receptor tyrosine kinase-like orphan receptor 1 (ROR1) [34], and toll-like receptor 4 (TLR4) [35] as potential receptors for resistin. However, both decorin and ROR1 are putative receptors for only murine resistin. Decorin and ROR1 were expressed at low levels in human mononuclear cells, and the expression was not increased by resistin treatment [36]. In human epithelial kidney cell line (HEK293), resistin competed with lipopolysaccharide (LPS) for binding to TLR4 and TLR4 mediated the pro-inflammatory effects of resistin [35], but there was no evidence for the direct interaction between TLR4 and human resistin due to the lack of biochemical binding assays.

Lee et al. [36] reported adenylyl cyclase-associated protein 1 (CAP1) as a functional receptor for human resistin and clarified resistin's intracellular signaling pathways in modulating inflammatory actions of monocytes. Human resistin directly binds to CAP1 of THP-1 (human acute monocytic leukemia cell line) monocytes and upregulates cyclic AMP (cAMP) concentration, protein

kinase A (PKA) activity, and nuclear factor-kappaB (NF-kB)-related transcription of inflammatory cytokines [36]. Overexpression of CAP1 in THP-1 monocytes enhanced the resistin-induced increased activity of the cAMP-dependent signaling [36]. Importantly, murine CAP1 was known to have 97 % homology with human CAP1 [37]. Infusion of CAP1-overexpressed monocytes aggravated adipose tissue inflammation in humanized mice lacking murine resistin but transgenic for a bacterial artificial chromosome containing human resistin (BAC-Retn) [38]. In contrast, suppression of CAP1 expression abrogated the resistin-mediated inflammatory activity both in vitro and in vivo. Therefore, CAP1 has been suggested as the bona fide receptor for resistin leading to inflammation [36].

Resistin Physiology and Pathophysiology

Resistin regulates glucose homeostasis, insulin resistance and inflammation, which are increasingly recognized to play pathogenic roles in obesity, diabetes, and cardiovascular diseases (Fig. 9.2).

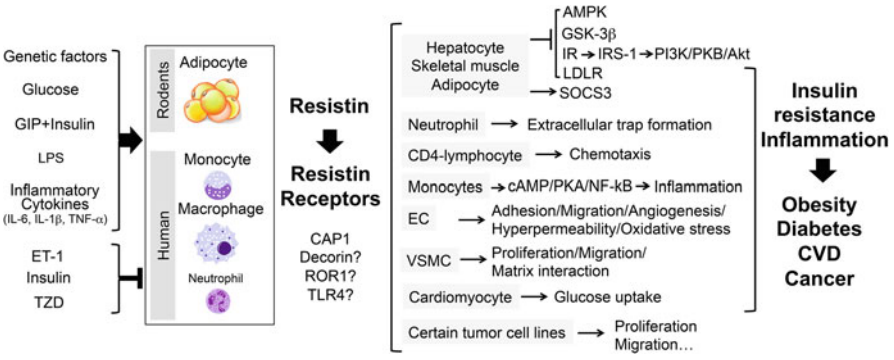

Fig. 9.2 Central role of resistin in obesity-associated disorders. Resistin is mainly expressed by adipocytes in rodents, while by monocytes, macrophages and neutrophils in human. Resistin is induced in response to various stimuli and genetically determined factors, but repressed by ET-1, insulin and TZD drugs. CAP-1 is a newly identified resistin receptor while decorin, ROR1, and TLR4 may also mediate resistin signaling. Resistin targets multiple cell types and signaling pathways to promote insulin resistance and inflammation, which are essential pathways linking obesity to diabetes, CVD and cancers. *AMPK*, AMP-activated protein kinase, *CAP1*, adenylyl cyclase-associated protein 1, *CVD* cardiovascular diseases, *EC* endothelial cells, *ET* endothelin, *GIP* glucose-dependent insulinotropic polypeptide, *GSK-3β* glycogen synthase kinase-3β, *IL* interleukin, *IR* insulin receptor, *IRS* insulin receptor substrate, *LDLR* low-density lipoprotein receptor, *LPS* lipopolysaccharide, *MAPK* mitogen-activated protein kinase, *NF-kB* nuclear factor-kappaB, *PI3K* phosphatidylinositol-3-kinase, *PKB* protein kinase B, *ROR1* tyrosine kinase-like orphan receptor 1, *TLR4* toll-like receptor 4, *TNF* tumor necrosis factor, *TZD* thiazolidinedione, *VSMC* vascular smooth muscle cell. "↑" indicates stimulation and "T" indicates inhibition

Resistin in Rodents

Several lines of evidence support that resistin plays a role in glucose metabolism in rodents. Resistin expression was upregulated in 3T3-L1 adipocytes by high glucose [39] and glucose-dependent insulinotropic polypeptide (GIP) in the presence of insulin [40], but was reduced by insulin [39], TZD drugs [23], and inflammatory cytokine tumor necrosis factor α (TNF-α) [41]. There were also conflicting results showing that insulin can stimulate resistin secretion in 3 T3-L1 adipocytes, and the effects were reversed by a vasoconstrictor endothelin (ET)-1 [42]. Thus, resistin expression is regulated by a variety of hormones and cytokines related to glucose homeostasis and metabolism.

Resistin secreted by adipocytes acts on hepatocytes, skeletal muscle myocytes, cardiomyocytes, and adipocytes themselves. In 3T3-L1 adipocytes, resistin attenuates multiple effects of insulin, including insulin receptor (IR) phosphorylation, IR substrate 1 (IRS-1) phosphorylation, phosphatidylinositol-3-kinase (PI3K) activation, phosphatidylinositol triphosphate production, as well as activation of protein kinase B (PKB)/Akt, and insulin-induced glucose uptake [16, 43]. Resistin treatment induces the gene expression of the suppressor of cytokine signaling 3 (SOCS-3), a known inhibitor of insulin signaling. Inhibition of SOCS prevented resistin from antagonizing insulin action in 3T3-L1 adipocytes [43]. Resisin increased the expression of GIP receptor and mimicked the effects of GIP on the PKB/LKB1/AMPK/LPL pathway by increasing phosphorylation of PKB, reducing levels of phosphorylated liver kinase B1 (LKB1) and AMP-activated kinase (AMPK), and increasing adipocyte lipoprotein lipase (LPL) activity [40, 44]. In primary rat hepatocyte, murine resistin reduces the levels of phosphorylated glycogen synthase kinase-3β (GSK-3β) at Ser 9, leading to impaired hepatic insulin action [45]. Recombinant murine resistin decreased insulin-stimulated glucose uptake into murine cardiomyocytes and rat skeletal muscle cells [26, 46, 47].

Resistin expression has been examined in different rodent models of obesity and diabetes. Steppan et al. reported that serum resistin levels were elevated in both diet-induced and genetically obese mice (*ob*/*ob* and *db*/*db*) [16]. The administration of recombinant resistin in wild type mice impaired glucose tolerance and insulin sensitivity [16], and suppressed phosphorylation of AMPK, a well-known metabolic sensor to regulate glucose and lipid metabolism [48], in liver, skeletal muscle, and WAT [49, 50]. Inhibition of resistin, conversely, led to an increase in the phosphorylation of AMPK [49]. Overexpressing resistin protein in male Wistar rats using intravenous administration of adenovirus encoding mouse resistin showed glucose intolerance, hyperinsulinemia, and impaired insulin sensitivity, as well as impaired IRS-1/IRS-2 phosphorylation and Akt activation in muscle and adipose tissue [50]. Both peripheral administration of recombinant resistin and transgenic overexpression of resistin induced hepatic insulin resistance in mice [16, 51, 52]. Conversely, neutralization of resistin with anti-resistin antibody, knockdown or deletion of resistin have been suggested to improve insulin sensitivity in liver, muscle, and WAT in either diet-induced obese mice or *ob*/*ob* mice [49, 52, 53]. In particular, resistin knockout mice showed

low glucose levels after fasting, which was associated with decreased expression of gluconeogenic enzymes in liver [52]. Genetic knockout of resistin [52] or a specific antisense oligodeoxynucleotide (ASO) directed against resistin mRNA [49] reversed the diet-induced hepatic insulin resistance, which was mainly attributable to a dramatic reduction in glucose production [52]. All of these findings suggest that resistin plays an important role in glucose homeostasis and insulin sensitivity in rodents. Beyond resistin's peripheral actions in liver, skeletal muscles, and adipose tissue, like other adipose-derived factors such as adiponectin and leptin, resistin may also act centrally to modulate food intake and energy expenditure. However, central administration of resistin only induced modest and transient satiety, without affecting body weight, suggesting limited central action by resistin [54].

There were also seemingly controversial findings on the expression and circulating levels of resistin in obese and diabetic animal models. Way et al. [55] firstly reported that resistin gene expression was significantly decreased in the WAT of several different murine models of obesity, including *ob*/*ob*, *db*/*db*, *tub*/*tub* and *KKA*(*y*) mice, compared with their lean counterparts. The secretion of resistin from the fat pads in *db*/*db* mice was significantly lower than that in lean mice, but serum levels of resistin were comparable between the obese and lean groups, perhaps due to the increased total fat mass in *db*/*db* mice [56]. In contrast, Rajala et al. suggested that serum levels of resistin in *db*/*db* mice were decreased [57]. Furthermore, in response to several different classes of antidiabetic PPAR-γ agonists, resistin gene expression in WAT was increased in both *ob*/*ob* and *db*/*db* mice [56] and Zucker obese rats [58]. And an improvement of insulin sensitivity after PPAR-α agonist treatment was accompanied by paradoxical increase of circulating resistin levels [58]. The reasons for the discrepancies remain unclear. One possible explanation for the reduced adipocyte resistin gene expression in murine models of obesity is that although the mRNA levels in the adipose tissue are lower [55], the increase in the total adipose tissue mass leads to an increase in the circulating levels of resistin protein. Another possibility is that, in obesity, either the stability or clearance of resistin is altered, leading to elevated levels despite decreased expression. Different assay methods may also render the results not comparable across studies. The controversial results and the potential limitation to use murine models of obesity to examine the role of resistin in human pathophysiology has led to further scientific research in human cell lines, humanized mice models and human subjects.

Resistin in Human

Human resistin is highly expressed in monocytes, and its expression is further increases when the mononuclear cells are differentiated into mature macrophages [29, 59]. Resistin is also expressed in neutrophil [60, 61], pancreatic islet [62], primary human bone marrow stem cells [63], mature human osteoblasts [63], rheumatoid arthritis synovial tissue [64], placenta trophoblastic cells [65], as well as certain tumor cell lines [66, 67]. Resistin gene expression in peripheral blood mononuclear

cells (PBMC) was robustly induced in response to various proinflammatory stimuli such as lipopolysaccharide (LPS) [68, 69], TNF-α [68], interleukin (IL)-6 [68], IL-1β [68] and resistin itself [70], suggesting an inflammatory role of resistin [68]. Resistin expression by monocyte-derived macrophage was also induced by LPS [59, 71]. This LPS induction in resistin was attenuated by immunoneutralization with a combination of antibodies neutralizing TNFα, IL-6, and IL-1β, suggesting that resistin secretion induced by LPS was mediated by secreted inflammatory cytokines that, in turn, induced resistin [59]. Resistin enhanced phosphorylation of Src and PI3K activation of CD4-positive lymphocytes and stimulated chemotaxis [72]. Resistin also enhanced neutrophil extracellular trap formation and proinflammatory activation [73]. In human primary and HepG2 hepatocytes, recombinant resistin inhibited low-density lipoprotein receptor (LDLR) levels via increasing cellular expression of proprotein convertase subtilisin/kexin type 9 (PCSK9), which enhanced intracellular LDLR lysosomal degradation [74].

In human study participants, experimental endotoxemia, which produces an insulin-resistant state, induced a remarkable rise in circulating resistin levels [59, 71]. TZD drugs downregulated the expression of human resistin in macrophages and reduced serum resistin levels [29, 59, 75]. Obese individuals who were likely to have greater infiltration of macrophages in adipose tissue showed increased expression of resistin in adipose tissue biopsy or had higher circulating levels of resistin than lean individuals [27, 59]. The effects of weight reduction on resistin levels in obese subjects, however, showed inconsistent results. Circulating resistin levels were reduced [76] or remained unchanged [77, 78] despite intervention of weight reduction program including diets and physical activity changes [76] or bariatric surgeries [77, 78]. Several reports also suggested that serum resistin levels were not associated with parameters of obesity or insulin resistance [71, 79, 80].

It is noteworthy that the inflammatory effects of human resistin are postulated to be involved in the development of insulin resistance, but the role of human resistin in the development of insulin resistance remains undetermined due to the lack of animal models with resistin expression from monocyte and macrophages as shown in human subjects. The Lazar group generated humanized mice lacking murine resistin but transgenic for a bacterial artificial chromosome containing human resistin (BAC-Retn) [38], in order to study the metabolic and molecular impact of human resistin in vivo. Results from these BAC-Retn mice showed that circulating resistin levels were within the normal range of that in human [38]. Similar to humans, LPS markedly increased serum resistin levels in the BAC-Retn mice [38]. Acute endotoxemia caused hypoglycemia in mice lacking murine resistin, but to less extent in BAC-Retn mice [38]. In addition, BAC-Retn mice developed severe hepatic insulin resistance under chronic endotoxemia, accompanied by increased inflammatory responses in liver and skeletal muscle [38]. These results strongly support the role of human resistin in the development of insulin resistance. In LPS-induced acute lung injury, humanized resistin mice demonstrated enhanced production of proinflammatory cytokines, more severe pulmonary edema, increased neutrophil extracellular trap formation, and elevated concentration of the alarmins HMGB1 and histone 3 in the lungs [73]. Those results suggest that human resistin play an important contributory role in inflammation and insulin resistance.

In addition to its role in inflammation and insulin resistance, resistin also affects functions of vascular cells, suggesting its potential role in atherogenesis. Indeed, resistin protein was expressed in human atherosclerotic lesions [81] and colocalized with lesion macrophages [28]. Resistin increased cell adhesion of THP-1 monocytes and human umbilical vein endothelial cell (HUVEC) by upregulating very late antigen-4 (VLA-4) (α4-, β1-integrin) on monocytes [82], and vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1 on endothelial cells [82] through a p38 mitogen-activated protein kinase (MAPK)-dependent pathway [83]. Resistin augmented monocyte infiltration in collagen by direct chemoattractive effect as well as by enhancing migration toward monocyte chemotactic protein-1 [82]. In HUVECs, resistin treatment increased ET-1 expression and plasminogen activator inhibitor (PAI)-1 release [83] downregulated endothelial nitric oxide synthase (eNOS) and upregulated inducible nitric oxide synthase (iNOS) expression, thereby reducing nitric oxide (NO) bioavailability and exacerbating oxidative stress and endothelial dysfunction [84]. In human coronary artery endothelial cells (HCAECs), resistin increased endothelial permeability, the principal event initiating lesion formation, through enhanced oxidative stress and the activation of p38MAPK [85]. Resistin also increased proliferation, migration and matrix interaction of human vascular smooth muscle cells, which are key pathological elements in atherogenesis [28, 86, 87].

Thus, human resistin may link insulin resistance and inflammatory status to the pathogenesis of obesity, diabetes, and associated cardiovascular diseases.

Genetic Studies of Resistin

Genetics of resistin expression and circulating levels has also drawn considerable research interest [88]. A number of studies examined whether expression and circulating levels of resistin are genetically controlled and the role of these genetic variants in subsequent clinical outcomes, including obesity, diabetes, cardiovascular diseases, and cancers. Resistin expression was associated with gene polymorphisms [89]. The -180C>G in the resistin gene led to increased basal promoter activity in adipocytes. The data were recapitulated in vivo, where G/G homozygotes had higher resistin mRNA levels in human abdominal subcutaneous adipose tissue adjusting for ethnicity and sex ($n=58$) [90]. The -638G>A, -420C>G, and -358G>A polymorphisms in the promoter region showed marked linkage disequilibrium, and were associated with serum resistin level in a Japanese population [91]. Additional studies suggested that -638G>A together with the -420C>G polymorphism influenced resistin gene transcriptional activity [91], partly through the specific recognition of -420G by Sp1/3, which increased resistin promoter activity [92]. The association of -420C>G (rs1862513, one of the most commonly studied polymorphisms) with resistin levels was also confirmed by a meta-analysis [93]. Another large-scale fine-mapping analysis of the resistin gene has found that single nucleotide polymorphisms (SNPs) including rs1477341, rs4804765, rs1423096, and

rs10401670 in the 3′ untranslated region (UTR) were associated with circulating resistin levels in a European population [93].

Polymorphisms in the resistin gene are also reported to be associated with metabolic abnormalities, but the results are inconsistent. Resistin gene polymorphism -420C>G was associated with body mass index (BMI) in a gender-specific manner and women carrying G allele had lower BMI compared to women carrying C/C homozygotes [94]. The -420C>G association with high resistin levels was related to progression of hyperglycemia, and insulin resistance in a Japanese [95] and southern Chinese population [96]. The data in other populations have been conflicting [6]. In a Caucasian cohort of nondiabetic subjects, -420C>G showed modest associations with plasma resistin and inflammatory biomarkers, but was not associated with BMI, fasting glucose, metabolic syndrome or coronary calcification [97]. In a Korea population, -420C>G determined resistin levels, but was not associated with obesity or type 2 diabetes [98]. Furthermore, -420C>G resistin polymorphism was not associated with either colorectal cancer [99] or endometrial cancer [100]. In a genome wide association study (GWAS) and quantitative trait loci (QTLs) study, two SNPs rs3745367 (intronic) and rs1423096 (in the 3′ UTR) on the resistin gene were associated with resistin levels in Han Chinese subjects with young-onset hypertensive. In addition, rs1423096 was significantly associated with metabolic syndrome and type 2 diabetes [101]. Although this study pointed to a causal association of resistin and type 2 diabetes, as only two SNPs on resistin gene were included in the Illumina HumanHap550 chip, fine-mapping and functional studies are needed to reveal genetic variants with stronger signal in the region.

Thus, molecular and genetics studies have suggested an association between resistin gene polymorphisms with resistin levels. However, genetic evidence for the association between resistin polymorphism and the risk of obesity, diabetes, and cancer remain inconclusive. Large-scale GWASs are required to further explore the causal association of resistin with obesity and related disorders.

Role of Resistin in Cancer: Epidemiological Studies

Human epidemiological studies have suggested the role of resistin in obesity [102, 103], insulin resistance [104–106], type 2 diabetes [76, 79, 102, 107–110], and cardiometabolic diseases [81]. A growing body of evidence supports a connection between obesity, diabetes and cancers, which share common risk factors and pathophysiological mechanisms. However, the relationship between resistin and cancers remains inconclusive [111]. Here we summarized current epidemiological studies examining associations between serum, plasma or tissue resistin levels and risks of various types of cancer (breast, prostate, colorectal, lung, gastric or gastroesophageal or esophageal, hematologic, renal, and pancreatic cancers) (Table. 9.1).

Table 9.1 Epidemiological studies on the relationship between resistin and cancer

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (*n*), Percentage of male (% male)	Results	Comments
Breast Cancer	Kang, 2007 [116]	Serum	Korea Case–control	Cases: Newly diagnosed breast cancer patients who were treated at a University Hospital ($n=41$, %male=0); Controls: age and body mass index (BMI)-matched ($n=43$, %male=0)	OR (95 % CI)=2.77 (1.40–5.50) comparing highest tertile vs. lowest tertile of resistin	Logistic regression model adjusting for age, BMI, menopause status, serum glucose and adiponectin level
	Hou, 2007 [115]	Serum	China Case–control	Cases: Newly diagnosed breast cancer patients who were treated at Qilu Hospital of Shandong University ($n=80$, %male=0); Controls: age matched healthy controls ($n=50$, %male=0)	OR (95 % CI)=1.334 (1.114–2.354, $p=0.012$)) for each 1-ng/ml increase of resistin level	Logistic regression model adjusting for adiponectin, leptin, BMI, waist circumference, triglyceride, total cholesterol, HDL-c, LDL-c and fasting blood glucose
	Gaudet, 2010 [120]	Serum	USA Nested case–control	Postmenopausal women in a cohort of women with prospectively collected serum samples obtained from 1977 to 2002 Cases: $n=234$, %male=0 Controls: $n=234$, %male=0	OR (95 % CI)=1.07 (0.62–1.83), $p_{trend}=0.85$, comparing highest quartile vs. lowest quartile of resistin	Conditional logistic regression models adjusting for age at reference, BMI, number of births, age at first full-term birth, years between blood collection and menopause and current postmenopausal hormone use

(continued)

Table 9.1 (continued)

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (*n*), Percentage of male (% male)	Results	Comments
	Sun, 2010 [117]	Plasma	Taiwan Hospital based case–control	Cases: women attending the Department of Surgery and health examination clinics at the Tri-Service General Hospital in Taipei, Taiwan from Jan 2004–Jun 2008 ($n=380$, %male=0); Controls: non-cancer women randomly recruited from individuals attending the health examination clinics in the same hospital, matched to each case by date of enrollment and duration of fasting ($n=760$, %male=0)	OR (95 % CI)=2.08 (1.04–3.85), $p_{trend}=0.03$, comparing highest quartile vs. lowest quartile of resistin	Conditional logistic regression models adjusting for age at enrollment, age at menarche, age at first full-term pregnancy, parity number, waist circumference, hormone replacement therapy use, and family history of breast cancer
	Lee, 2012 [119]	Tissue	Taiwan Cross-sectional	Newly diagnosed and surgically treated breast cancer patients at the Cancer Center of Kaohsiung Medical University Hospital during 2003–2008 ($n=110$, %male=0)	HR (95 % CI)=7.17 (1.74–29.60, p=0.006) for disease-free survival, and HR (95 % CI)=6.47 (1.22–34.25, p=0.028) for overall survival comparing high (>50 % positively stained cells) vs. Low resistin (<50 % positively stained cells) expression	Multivariable Cox regression models adjusting for tumor grade, age at diagnosis,, BMI, ER status, PR status, Her2/Neu status, radiotherapy, chemotherapy, and hormone therapy Chi-square test showed high resistin expression was correlated with tumor stage ($p=0.021$), tumor size ($p=0.008$), lymph node metastasis ($p=0.028$) and ER status ($p=0.042$)

	Dalamaga, 2013 [118]	Serum	Greek Case–control	Postmenopausal women aged 85 years or younger from the Veterans' Administration General Hospital of Athens Cases: $n=102$, %male=0 Controls: $n=102$, %male=0	OR (95% CI)=1.17 (1.03–1.34; $p=0.02$) for each 1-ng/ml increase of resistin level	Multivariable analyses adjusting for age, date of diagnosis, education, family history of cancer, exogenous hormones use, alcohol, smoking, physical activity, adipokines, reproductive, metabolic, anthropometric, and inflammatory markers
	Alokail, 2013 [121]	Serum	Saudi Arabia Case–control	Cases: newly diagnosed breast cancer ($n=56$, %male=0); Controls: age and BMI-matched ($n=53$, %male=0)	OR (95% CI)=1.9 (0.62–5.7; $p=0.26$) for each 1-ng/ml increase of resistin level	Multinomial logistic regression adjusting for menopausal status and age of menarche
	Stewart, 2013 [122]	Gene	USA Cross-sectional	Age and state-matched African American (AA) ($n=53$) and Caucasian American (CA) breast cancer patients ($n=574$, %male=0)	Log2 fold change for Gene RET$n=2.25$, adjusted $p=2.80E-03$ comparing mean AA expression vs. mean CA expression	Nest generation sequencing (NGS) data from the Cancer Genome Atlas (TCGA) was used. DESeq version 1.10.1 was employed to test for different gene expression using R programming
Prostate Cancer	Parekh, 2007 [130]	Serum	USA Case–control	Cases: incident prostate cancer cases from the San Antonio Center for Biomarkers of Risk of Prostate Cancer cohort study ($n=123$, %male=100%); Controls: age-matched with cases from the same cohort ($n=127$, %male=100%)	OR=0.82, $p=0.10$ comparing resistin level greater than the medial value of controls vs. not	Univariate logistic regression. was used, Bonferroni adjustment was conducted accounting for multiple comparisons
	Smith, 2008 [132]	Plasma	USA Cross-sectional	25 men with locally advanced or recurrent prostate cancer and were treated with leuprolide depot and bicalutamide ($n=25$, %male=100%)	Resistin level did not change significantly comparing baseline (16.1 ±0.8 ng/ml) and week 12 levels (15.2±0.9 ng/ml), $p=0.74$	Two-sided paired *t*-test was used to examine the significance of the changes

(continued)

Table 9.1 (continued)

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (*n*), Percentage of male (% male)	Results	Comments
	Housa, 2008 [131]	Serum	Czech Republic Cross-sectional	Men referred to simple prostatectomy for benign prostate hyperplasia (BPH) ($n=26$) or radical prostatectomy for prostate cancer [$n=18$ (pT2, organ confined) and $n=24$ (pT3, advanced)], %male = 100 %	Mean ± SD of serum resistin (ng/ml) was 7.09 ± 2.83 in BPH patients, was 7.33 ± 4.98 in prostate cancer patients ($p=0.34$)	Independent two-sample *t*-test was used. Stratified by prostate cancer stage, no significant difference of resistin level was observed between pT2 and pT3 groups Additional analyses did not show significant association between prostate tissue resistin immunohistochemistry level and histological grade of tumor
Colorectal Cancer (CRC) or Colon Cancer (CC)	Wagsater, 2008 [99]	Plasma, gene, tissue	Sweden Case–control	Cases: patients from southeastern Sweden who underwent surgical resections for primary colorectal adenocarcinoma at a local hospital $n=248$ ($n=124$ for colon, $n=124$ for rectum), %male = 51 %; Controls: $n=256$ from the same geographical region as the CRC patients, %male = 53 %	Median (range) of plasma resistin in case was 3950 pg/ mL (1500–24,000 pg/ml) and in control was 5250 pg/ml (2000–30,000 pg/ml) ($p=0.68$). Protein expression of resistin in tumor (median: 1755 pg/ml) vs. normal tissue (median: 269 pg/mg) showed significant difference ($p<0.0001$) The resistin polymorphism (-420C>G) does not show significant difference between cases and controls	Mann–Whitney *U* test was used to compare differences in resistin plasma level. Wilcoxon's signed rank test was used to compare resistin protein level in tissues. Chi-square test was sued to compare genotype distribution and the Hardy-Weinberg equilibrium was tested

	Kumor, 2009 [136]	Serum	Poland Case–control	Patients with adenomatous polyps ($n=37$, %male = 38 %) and CRC ($n=36$, %male = 47 %) Controls: $n=25$, %male = 40 %	Mean ± SD of serum resistin (ng/ml) was 3.6 ± 1.08 in control group, 4.89 ± 2.15 in low grade (LGD) adenoma group, 5.7 ± 2.89 in high grade (HGD) adenoma group, 5.86 ± 3.1 in adenocarcinoma low grade CRC group, 6.79 ± 2.41 in high grade CRC group.(LGD adenoma compared to control, $p<0.05$)	Comparisons between groups were made with *t*-test or Mann–Whitney rank-sum test. Resistin level in adenoma or CRC groups was significantly higher than control group. No significant difference between adenoma and CRC group
	Nakajima, 2010 [137]	Blood (source unspecified)	Japan Case–control	Cases: newly diagnosed CRC patients who had undergone upper total colonoscopy at the hospital Feb 1999-Feb 2007 ($n=115$, %male = 60 %) Controls: Age-, sex, and BMI matched controls identified from those patients undergoing colonoscopy and were free from CRC or adenoma ($n=115$, %male = 60 %)	OR (95 % CI) = 2.067 (1.053–4.055), $p=0.03$ each log (1-ng/ml) increase of resistin level Mean ± SD of log (resistin) was 1.2 ± 0.5 in control group, 1.3 ± 0.5 in Stage 0 group, 1.7 ± 0.5 in Stage 4 group ($p<0.01$)	Multivariate logistic regression model was used to estimate OR (95 % CI); one-way ANOVA was used to compare resistin levels between multiple groups
	Otake, 2010 [143]	Fasting plasma	Japan Case–control	Males aged at least 31 years undergoing colonoscopy examination who were confirmed having adenoma or cancer in the hospital were identified for cases ($n=47$ with adenoma, $n=34$ with early cancer, $n=17$ with advanced cancer, %male = 100 %), confirmed lack of polyps of the entire colon and rectum were identified as controls ($n=26$, %male = 100 %);	OR (95 % CI) = 1.184 (0.524–3.642), p = 0.514 for adenoma; 0.980 (0.324–2.808), p = 0.969 for early cancer; 0.667 (0.190–2.334), $p=0.526$ for advanced cancer comparing resistin (ng/mL) ≥ 42 vs. < 42	Univariate logistic regression was used

(continued)

Table 9.1 (continued)

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (*n*), Percentage of male (% male)	Results	Comments
	Al-Harithy, 2010 [138]	Serum, gene	Saudi Arabia Case–control	Colon cancer patients ($n=60$, %male = 52 %) and gender & age-matched controls ($n=60$, %male = 50 %)	Mean ± SD of serum resistin (ng/ml) was 19.44 ± 8.46 in case group, and 5.45 ± 2.73 in control group.($p=0.0001$) For SNP C-180G genotype, CG and GG genotype carriers had higher serum resistin compared to CC genotype carriers in patients ($p=0.08$), no difference in control group	*t*-test was used to compare continuous mean serum resistin level. Chi-square test was used to compare genotypes distribution and the Hardy- Weinberg equilibrium was tested
	Gonullu, 2010 [139]	Fasting serum	Turkey Case–control	Cases: CRC patients ($n=36$ with $n=12$ metastatic, %male = 50 %) Control: healthy volunteers who had colonoscopy for various reasons ($n=37$, %male = 54 %)	Mean ± SD of serum resistin (ng/ml) was 6.1 ± 3.3 in case group, and 4.5 ± 1.5 in control group.($p=0.026$) Correlation between diseases stage and resistin ($p=0.534$)	Kruskal–Wallis test and the Mann–Whitney *U* test was used to compare biomarkers between groups. Spearman-rho test was used for correlation tests
	Salageanu, 2010 [140]	Serum	Romania Case–control	Cases: colon cancer patients who had surgery at Emergency University Hospital Bucharest ($n=29$, %male = 59 %) Controls: healthy volunteers ($n=27$, %male = 44 %)	Data represented in figure. Resistin levels in colon cancer patients before surgery and after surgery were both statistically significantly different from controls ($p<0.05$)	Two-tailed Wilcoxon signed-rank test was used

	Ho, 2012 [145]	Fasting plasma	USA Case-cohort	Cases: women enrolled in the Women's Health Initiative Observational (WHI-OS) studies who developed an incident primary colon or rectal cancer by June 2004 after the first year of follow up ($n=457$ with $n=373$ colon cancer, $n=82$ rectosigmoid junction or rectal cancer, $n=2$ unknown location); Subcohort: randomly sampling subjects from the WHI-OS who had >12 month follow-up and no history of breast, CRC or endometrial cancer at 12 months ($n=841$, %male = 0 %)	HR (95 % CI) = 0.98 (0.68–1.43, $p=0.994$) for CRC risk comparing highest quartile (≥15.8 ng/ml)) vs. Lowest quartile (<10.0 ng/ml) of resistin level	Cox proportional hazard regression model with robust variance estimation using the Self-Prentice methods adjusting for age, race, smoking, ever had colonoscopy, estrogen level, insulin and waist circumference
	Hillenbran, 2012 [144]	Fasting plasma for CRC and morbidly obese (MO) patients, non-fasting plasma for healthy blood donors	Germany Case–control	Cases: CRC ($n=31$ rectal cancer and $n=36$ colon cancer, %male = 63 %) and MO patients ($n=37$, %male = 35 %) Controls ($n=60$, %male = 50 %)	Median (range) of resistin (ng. ml) was 10.9 (4.2–43.5) among CRC, 9.9 (3.7–83.6) among MO and 10.8 (5.2–24.9) among controls. All pairwise group comparisons $p>0.05$. No correlations between resistin and tumor stage among colon cancer and rectal cancer patients	Mann–Whitney test was used Spearman's correlation test was used for correlation tests No adjustments were made for multiple statistical comparisons

(continued)

Table 9.1 (continued)

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (*n*), Percentage of male (% male)	Results	Comments
	Danese, 2012 [141]	Fasting serum	Italy Case–control	Cases: CRC patients who had biopsy-proven CRC among patients who had total colonoscopy at the University hospital ($n=40$, %male = 53 %); Controls: age, sex and BMI-matched controls free from CRC or adenoma by colonoscopy ($n=40$, %male = 65 %)	Median (range) of resistin (ng/ml) was 5.88 (1.8–22.3) in case group, and 4.94 (1.8–8.2) in control group ($p<0.05$). OR (95%CI) of CRC per 1 unit increase of resistin was 1.331 (1.030–1.719) Resistin levels increased with tumor stage increased	Two-sample *t*-test was used for comparing resistin between the two groups. Logistic regression adjusting for age, sex, BMI and lifestyle parameters were used for calculating OR C-reactive protein (CRP) was found to be significantly associated with resistin
	Kosova, 2013 [147]	Fasting serum	Turkey Cross-sectional	Convenience sample of patients with colon cancer ($n=20$ with $n=13$ who had treatment of chemo- and radio-therapy, %male = 60 %) and patients with benign anal disease at the outpatient department of a local hospital ($n=20$ who had operation, %male = 45 %)	Mean ± SD of resistin (ng/ml) was 4.92 ± 2.2 in colon cancer group before treatment, 5.96 ± 2.8 in colon cancer group after treatment, 3.39 ± 1.1 in benign group before treatment, 5.62 ± 1.7 in benign group after treatment	Mann–Whitney *U* test and Wilcoxon test were used comparing the groups. Significant difference observed between colon cancer group and benign group before treatment. The levels after treatment were not statistically significantly different
	Slomian, 2014 [146]	Fasting serum	Poland Prospective	Patients with pathologically documented advanced CRC and measurable metastatic disease who required palliative chemotherapy and had either partial response or stabilization ($n=28$, %male = 43 %)	Mean ± SD of resistin (ng/ml) was 7.24 ± 1.17 before chemotherapy and 6.36 ± 1.36 after chemotherapy ($p<0.001$) with change (%) = −25 %	Student *t*-test, *t*-test with separate variance estimate, Mann–Whitney *U* test or ANOVA Kruskal–Wallis test was used. No gender difference

	Joshi, 2014 [142]	Fasting serum	Korea Case–control	Cases: patients newly diagnosed with CRC from Keimyung University Hospital ($n=100$, %male = 60 %); Controls: age and sex matched individuals from Kangwon National University Hospital who received negative diagnoses of CRC by serological blood test ($n=100$, %male = 60 %)	Mean ± SD of resistin (ng/ml) was 4.9 ± 2.3 in case group, and 2.8 ± 1.7 in control group ($p<0.0001$). OR (95%CI) of CRC comparing high (above median) vs. low (below median) resistin was 6.08 (3.23–11.44) $p_{trend}<0.0001$	*t*-test was used to compare the mean of resistin between groups Unconditional logistic regression model adjusting for age, sex, and covariates including selected adipokines
Gastroesophageal cancer (GEC) or Gastric Cancer (GC) or Esophageal Cancer (EC)	Kerem, 2008 [157]	Fasting serum	Tukey Case–control	Cases: Patients with a pathological diagnosis of gastric adenocarcinoma were included, patients with gastric lymphoma and malignant stromal tumors were excluded (Non-cachexia: $n=30$, %male = 84 %; Cachexia: $n=23$, %male = 77 %) Controls: Healthy people over 40 years old ($n=30$, %male = 67 %)	Mean of resistin (ng/ml) was 43.4 in noncachexia group, 66.7 in cachexia group, 18.1 in control group ($p<0.001$)	ANOVA was used to compare resistin levels between groups
	Diakowska, 2014 [156]	Fasting serum	Poland Case–control	Cases: GEC patients hospitalized in the Department of Gastrointestinal and General Surgery of Wroclaw Medical University Apr 2008–Dec 2012 ($n=85$, %male = 73 %) Controls: apparently healthy individuals ($n=60$, %male = 78 %)	Mean ± SD of resistin (ng/ml) was 8.99 ± 3.21 in GEC noncachectic group, 11.74 ± 2.98 in GEC cachectic group and 7.5 ± 2.7 in control ($p<0.001$). Cachectic group was significantly from either control or noncachectic group	One-way ANOVA with Tukey's post hoc test

(continued)

Table 9.1 (continued)

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (*n*), Percentage of male (% male)	Results	Comments
	Nakajima, 2009 [154]	Blood	Japan Case–control	Cases: pathologically diagnosed by biopsy through upper gastrointestinal (UGI) endoscopy and newly treated at the National Cancer Center Hospital in Tokyo ($n=156$, %male = 58 %) Controls: age- and sex-matched controls free from GC by UGI endoscopy ($n=156$, %male = 58 %)	Resistin (ng/ml) was significantly higher in GC patients (median: 5.1) compared to controls (median: 3.0) ($p=0.0004$). OR (95 % CI) per 1 unit increase of resistin was 4.548 (1.926–10.740), $p=0.0006$ Resistin level increased with stage increased ($p<0.0001$)	Two-sample *t*-test was used for continuous variables comparing patients and controls group. Multivariate conditional logistic regression was used
	Nakajima, 2010 [155]	Blood	Japan Case–control	Cases: pathologically diagnosed squamous cell carcinoma of esophagus (SCCE) by biopsy using gastrointestinal (UGI) endoscopy ($n=117$, %male = 74 %) Controls: age- and sex-matched subjects free from SCCE by UGI endoscopy ($n=117$, %male = 74 %)	Resistin levels in SCCE patients (median = 4.7 ng/ml) were higher than those in the controls (median = 3.0 ng/ml) ($p<0.01$) OR (95 % CI) per 1 unit increase of resistin was 3.43 (1.66–7.06), $p<0.01$ Resistin level also progressed with stage progression ($p<0.01$)	Conditional logistic regression models were used to derive OR. One-way ANOVA was used to examine resistin level between tumor stage groups

Lung cancer	Karapanagiotou, 2008 [148]	Fasting serum	Greece Case–control	Cases: advanced non-small cell lung cancer patients ($n=101$, %male = 82 %) Controls: healthy volunteers ($n=51$, %male = 51 %)	Mean ± SD of resistin (ng/ml) was 5.0 ± 4.0 in case group, and 3.5 ± 1.4 in control group. ($p=0.007$).	Two-sample t-test was used for comparison and the p-value was adjusted for age, sex, and BMI Increasing resistin level was associated with weight loss ($p=0.007$) through Spearman's correlation tests
	Kuo, 2013 [67]	Preoperative blood serum	Taiwan Case–control	Cases: lung cancer patients ($n=46$) admitted to the Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Controls: healthy donors ($n=24$)	Mean of resistin (pg/ml) was 1779.04 in case group, and 843.86 in control group, $p=0.0136$ (results presented in figure)	Student's t-test was used to compare resistin level between groups
Hematologic Malignancies	Pamuk, 2006 [160]	Serum	Turkey Case–control	Cases: patients with lymphoma ($n=21$), multiple myeloma (MM) ($n=14$), acute leukemia ($n=14$), chronic lymphocytic leukemia (CLL) ($n=13$); Controls: healthy subjects ($n=25$)	Mean ± SD of resistin (ng/ml) was 3.94 ± 3.4 in lymphoma group, 1.9 ± 0.8 in CLL group, 2.04 ± 1.2 in acute leukemia group, 3.3 ± 3.3 in MM group, and 1.97 ± 0.6 in control group. Resistin level is significantly higher in lymphoma than CLL, acute leukemia, and control group ($p<0.01$)	One-way variance analysis (ANOVA) was used and pair-wise comparisons between each group were conducted by Tukey test. Correlation analysis was conducted using Pearson's correlation test Resistin correlated with platelet count in patients with hematological malignancies ($r=0.26$,$p=0.04$)

(continued)

Table 9.1 (continued)

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (*n*), Percentage of male (% male)	Results	Comments
	Dalamaga, 2008 [161]	Serum	Greece Case–control	Cases: Newly diagnosed myelodysplastic syndromes (MDS) patients under age 83 who were admitted to the Veterans' hospital ($n=101$, %male = 58 %); Controls: Patient under age 83 admitted for nonneoplastic and noninfectious diseases to the Orthopedic and Ophthalmologic Department of the same hospital and were matched to cases on age, gender and year/month diagnosis ($n=101$, %male = 58 %).	OR (95 % CI) =0.20 (0.07–0.51), $p<0.001$ for MDS comparing the highest (range 35.8–185.5 ng/ml) vs lowest quartile of resistin (range 0.5–17.3 ng/ml)	Unconditional logistical regression model adjusting for age, gender, height, weight, BMI, high molecular weight, leptin, adiponectin, IGF-1 and IGFBP-3
	Dalamaga, 2009 [162]	Serum	Greece Case–control	Cases: Newly diagnosed multiple myeloma patients under age 79 years from the Veterans' hospital ($n=73$, %male = 58 %); Controls: Age, gender and year/ month of diagnosis-matched patients under 79 years old who were admitted to the same hospital for nonneoplastic and noninfectious conditions to the Ophthalmologic and Orthopedic Department ($n=73$, %male=58 %)	OR (95 % CI) =0.03 (0.004–0.17), $p<0.001$ for multiple myeloma comparing the highest (median 24.7 ng/ml) vs lowest quartile of resistin (median 7.4 ng/ml)	Unconditional logistical regression adjusted for age, gender, date of diagnosis, BMI, leptin, and adiponectin levels

	Reseland, 2009 [163]	Plasma	Norway Case–control	Cases: Newly diagnosed multiple myeloma patients ($n=23$, %male=52 %); Controls: Healthy people matched on age and BMI ($n=23$, %male=57 %)	There was no significant difference in resistin levels between cases and controls (data shown in figure)	Plasma resistin levels were presented as whisker and box plots. Mann–Whitney U rest or the Wilcoxon signed rank test was used
	Moschovi, 2010 [158]	Fasting plasma	Greece Case–control and longitudinal	Cases: Children (age 4.3±2.1 years at baseline) with newly diagnosed B-cell acute lymphoblastic leukemia (ALL) ($n=9$, %male=56 %); Controls: healthy children matched for age, sex, and BMI ($n=9$, %male=56 %)	Mean±SD of resistin (ng/ml) was 5.2±1.2 in ALL group and 3.2±1.1 in control group ($p<0.001$). Comparing resistin levels between baseline (5.2±1.2 ng/ml) and before the end of maintenance phase (3.4±0.9 ng/ml), resistin level became significantly lower ($p<0.001$)	Student *t*-test was used to compare resistin levels in case and control groups. Paired student *t*-test was used to compare data within groups
	Kohler, 2011 [159]	Fasting blood	UK	Cases: Children>6 years with acute lymphoblastic leukemia (ALL) without cranial radiotherapy treatment ($n=54$, %male=50 %); Controls: healthy children who had never received steroid treatment and no other chronic medical conditions ($n=51$, %male=41 %)	Among females, mean±SD of blood resistin (ng/ml) was 16.0±8.8 in case group, and 16.3±10.3 in control group ($p=$NS); among males, mean±SD of blood resistin (ng/ml) was 12.8±8.7 in case group, and 14.2±9.8 in control group. ($p=$NS)	Independent *t*-test was used to test statistical difference between ALL survivors and controls
	DiCarlo, 2014 [192]	Serum	USA Case–control	Cases: children 0–18 years receiving allogeneic or autologous hematopoietic stem cell transplantation ($n=51$, %male=59 %); Controls: healthy subjects' ages 13–40 years ($n=11$)	Mean±SD of serum resistin (pg/ml) was 643±678 in case group, and 1290±653 in control group. ($p=0.005$)	Unpaired two sample *t*-test was used for comparison

(continued)

Table 9.1 (continued)

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (n), Percentage of male (% male)	Results	Comments
Endometrial Cancer	Hlavna, 2011 [100]	Fasting serum; Gene -420 G/C (rs3219175)	Czech Republic Case–control	Cases: Caucasian women diagnosed with endometrial cancer and was treated at a university hospital ($n=37$, %male=0); Controls: healthy women matched on age and BMI ($n=37$, %male=0)	Mean of serum resistin (pg/ml) was 23.50 in case group and 9.44 in control group. ($p<0.01$). Genotype distribution was not statistically significant ($\chi=0.567$, $p=0.75$). No significant associations were observed between resistin and BMI, type 2 diabetes, hypertension or smoking	Nonparametric statistical methods were used due to non-normal distribution of the evaluated parameters. Mann–Whitney test was used for dichotomic outcomes; Kruskal–Wallis ANOVA was used for continuous outcomes and categorical outcomes with more than two categories
	Ilhan, 2015 [152]	Serum	Turkey Case–control	Cases: endometrial cancer patients identified from the Gynecologic Oncology Department of the University of Selcuk, Konya in Turkey; Controls: identified from healthy volunteers who went to the same clinic as cases for abnormal bleeding	No significant difference between cases and controls in serum resistin level (numbers not reported) OR (95 % CI) for lymph node metastasis per 1 unit increase of resistin was 1.018 (1.00–1.035, $p=0.046$)	t-test was used to compare case–control resistin levels. Logistic regression analysis was performed assessing resistin level and risk of lymph node metastasis
Pancreatic Cancer (PanC)	Al-Salam, 2011 [166]	Tissue	United Arab Emirates Case–control	Pancreatic tissue obtained after pancreatectomy due to PanC. Diabetes patients; ($n=3$, all female), Non-diabetes patients: ($n=2$, 1 male, 1 female)	From immunohistochemistry (IHC) analysis, resistin positive cells in pancreatic islets were higher in diabetes pancreas compared to control ($p<0.0001$). The number of cells positive for both resistin and insulin was significantly higher in diabetic pancreas than controls ($p<0.05$)	Resistin was not found to co-localize with glucagon in IHC analysis

Cancer type	Study (Author/year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (n), Percentage of male (% male)	Results	Comments
	Jiang, 2012 [167]	Tissue	China Cross-sectional	Patients with pancreatic ductal adenocarcinoma who underwent radical surgery in Hua Dong Hospital, China during 2006–2007 ($n=45$, %male = 60 %)	Significant difference of immunohistochemistry analysis of resistin expression was observed in poorly (100 %), moderately (39.3 %), and well (25 %) differentiated tumors ($p<0.01$). Relapse-free survival was significantly different between patients with (median, 9 months) and without (median, 18 months) resistin-stained tumors ($p<0.05$)	t-test and Chi-square test were used for group comparisons. Kaplan–Meier method was used for survival analyses
	Gasiorowska, 2013 [168]	Fasting Plasma	Poland Case–control	Cases: patients with PanC ($n=45$, %male = 40 %) Controls: age, gender and BMI matched healthy persons ($n=13$)	Mean ± SD of resistin (ng/mL) was 126.2 ± 143.2 in case group, 18.9 ± 7.2 in control group. ($p<0.009$) Median (range) of resistin (ng/mL) was 140.4 (4.8–343.2) in PanC patients with diabetes, 116.7 (27–415.2) in PanC patients without diabetes. ($p=0.59$)	Student's t-test was used for comparing continuous variables between groups No association was found between resistin level and tumor size, lymph node metastases, distant metastases, jaundice or pain intensity in the PanC group

(continued)

Table 9.1 (continued)

Cancer type	Study (Author/ year)	Biological Sample source for resistin	Country and study design	Population and number of subjects (*n*), Percentage of male (% male)	Results	Comments
Renal Cancer	Liao, 2013 [164]	Fasting serum	Finland Nested case–control study	Cases: incident renal cell carcinoma (RCC) subjects in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study of Finnish male smokers and provided a blood sample at the baseline visit ($n=273$, %male = 100 %); Controls: randomly selected from ATBC cohort and matched on age and date of baseline blood collection ($n=273$, %male = 100 %)	OR (95 % CI) = 1.27 (0.75–2.15), $p_{trend}=0.45$, comparing highest quartile vs. lowest quartile of resistin	Conditional logistic regression models adjusting for number of years smoking, presence of hypertension, history of diabetes, and physical activity. Tests for trend were calculated by modeling the median value within each category

Resistin and Breast Cancer

In the USA, breast cancer (BC) is the most common cancer and the second leading cause of cancer-related death among women [112]. Central adiposity is associated with postmenopausal BC [113], yet the body fat distribution is not associated with premenopausal BC [114]. Studies examining circulating resistin levels with overall BC risk have been inconclusive. Three case–control studies [115–117] including both premenopausal and postmenopausal women, and one case–control study [118] including only postmenopausal women showed high levels of resistin was associated with increased BC risk. A cross-sectional study including only newly diagnosed and surgically treated BC patients showed higher resistin level was associated with poor disease-free and overall survival [119]. Higher resistin levels also correlated with worse tumor stage, larger tumor size, lymph node metastasis, and estrogen receptor-positive status [119]. In contrast, a nested case–control study [120] including postmenopausal women and another case–control study including both premenopausal and postmenopausal women [121] did not find significant association between resistin and BC, both taking into account of BMI. In terms of racial difference in resistin gene expression, a study using BC data in the Cancer Genome Atlas (TCGA) showed four times increased expression of resistin gene among African American (AA) compared to Caucasian American (CA) BC patients, along with the other 673 genes and transcripts identified to be different between AA and CA [122]. This study also showed the higher the BC stage, the higher the gene expression diversity [39].

Higher blood estrogen level has been reported to be associated with both premenopausal and postmenopausal BC [123, 124], however, the association between resistin and estrogen levels showed inconsistent results. Sun et al. showed women who had higher levels of estrogens exposure had higher resistin levels [117]. On the other hand, Gaudet et al. did not find any association between resistin and estradiol levels [120]. This was supported by a study by Chalvatzs et al., which showed resistin levels were not affected among women receiving estrogen treatment of ovariectomy procedure, suggesting that ovarian hormones including estrogen did not regulate resistin production [125].

Although obesity has been suggested as an established risk factor for postmenopausal BC, the association between resistin level and BC stratified by menopausal status is still unclear. Future epidemiological studies are needed to stratify the menopausal status to better understand the association between resistin and BC risk.

Resistin and Prostate Cancer

Prostate cancer (PCa) is the most common non-skin cancer and the second leading cause of cancer-related death in men in the USA [112]. Obesity is one of the risk factors that has been studied extensively in association with PCa [126]. Several

studies have found body mass index (BMI), a surrogate of general adiposity, was positively associated with PCa, while other studies did not show any association [127]. A pooled meta-analysis of prospective studies showed that BMI was associated with decreased risk of local PCa but increased risk of advanced PCa [128]. On the other hand, another meta-analysis reported that higher waist circuference was associated with higher risks of PCa, but no association was found between BMI and PCa risks [129].

In terms of the association between resistin and PCa, an age-matched case–control study with 123 pairs found serum levels of resistin were underexpressed among PCa patients [130]. However, another hospital-based study including 26 patients with benign prostatic hyperplasia (BPH) and 42 patients with different stages of PCa reported that resistin was expressed at a higher level in high-grade PCa tissues than in low-grade PCa and BPH tissues [131]. This study also found a decreased prostate-specific antigen (PSA) level among PCa patients who had lower plasma resistin concentrations [131]. Moreover, a small-scale study among 26 recurrent or locally advanced PCa patients with gonadotropin-releasing hormone (GnRH) agonists therapy showed there was no significant change in resistin level over the course of 12 months of treatment [132]. In addition, another small-scale study among 25 PCa patients with leuprolide depot and bicalutamide treatment showed no significant change of resistin during a 12-week prospective study [133].

From the above human studies, the associations between resistin levels and PCa risk remain inconsistent, but it appears that resistin levels showed no significant change during the period of PCa treatment.

Resistin and Colorectal Cancer or Colon Cancer

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer-related death in both men and women [112]. It is also one of the obesity-related cancers. Central adiposity as well as general obesity has been found to be positively related to colorectal cancer [134]. Resistin is associated with CRC likely through obesity-driven inflammation [135]. The majority of the case–control studies (eight case–control studies) found a positive association between resistin and CRC [99, 136–142]. Two case–control studies [143, 144] and one case-cohort study [145] did not report significant association. A prospective study reported that chemotherapy reduced resistin levels in CRC patients [146], consistent with a cross-sectional study examining pre-treatment and post-treatment resistin levels showing higher resistin level in the malign group than that in the benign group before treatment while no difference was found between the two groups after treatment [147]. The latter study also suggested resistin may be used as a marker during the preoperative period rather than the postoperative period [147]. Only one study assessed gene polymorphism in association with CRC and it showed that the genetic distributions of −420C>G resistin polymorphism were not significantly different between patients and controls [99].

CRC is the most studied form of cancer in association with resistin. With most studies reporting significant associations, future molecular studies are needed to delineate the biological mechanisms and genetic susceptibility.

Resistin and Lung Cancer

Lung cancer is not an obesity-associated cancer and thus there have been limited research efforts investigating the association between resistin and lung cancer. Karapanagiotou et al. conducted a prospective study including 101 non-small cell lung cancer patients and 51 healthy control volunteers in Greece, and reported that serum resistin level was higher in patients than in controls ($p=0.007$) [148]. This study also suggested that resistin, rather than adiponectin or leptin, was associated with weight loss among these lung cancer patients ($p=0.007$) and deteriorating performance ($p=0.015$) [148]. A mechanisms study showed lung tumor associated dendritic cells (TADCS) derived resistin promote lung cancer progression through Twist pathway mediated by a histone methyltransferase, Wolf–Hirschhorn syndrome candidate 1 (WHSC1) [67]. More studies are needed to confirm these findings.

Resistin and Endometrial Cancer

Endometrial cancer (EnC) is the most common gynecological cancer and the fourth most common cancer among women in the USA. Incidence has increased over the past years [149, 150] with an estimated 54,870 newly diagnosed cases in 2015 [112]. Obesity and abdominal adiposity have been considered as major risk factors [112]. A recent meta-analysis pooling 40 studies involving 32,281,242 women confirmed the strong association between obesity and EnC risk, showing an ~1.5-fold increased risk in overweight women and more than 2.5-fold increased risk in obese women compared to nonobese women [151]. It was hypothesized that increased estrogen might be the driving force for this increased risk. Only two studies have been published to date examining the association between resistin and EnC. One study involving 37 pairs of matched patients and healthy controls in Czech Republic showed positive association between resistin levels and EnC, although genetic variants (-420C>G) did not contribute to this difference [100]. However, the other study among 43 EnC patients and 42 controls did not detect significant association; instead, this study found resistin level was associated with higher risk of lymph node metastasis, suggesting resistin being a predictor for advanced stage of EnC [152].

Resistin and Gastric Cancer/Gastroesophageal Cancer

Despite the overall decrease in the incidence of gastric cancer (GC) and esophageal cancer (EC), GC and EC still remain as leading causes of cancer death in less developed countries [153]. Smoking and infection have been the major contributing factors for these cancers [153]. The association between resistin and GC or EC has been less investigated. A study including 156 GC patients and controls in Japan found positive association between GC and blood resistin levels, and the higher the resistin level, the more advanced the cancer [154]. The same study group in Japan also reported similar associations between blood resistin level and squamous cell carcinoma of the esophagus as well as the stage progression [155]. Another study in Poland among 85 gastroesophageal cancer (GEC) patients who were classified into GEC cachectic and GEC non-cachectic groups showed a significant higher resistin level among GEC cachectic patients compared to GEC non-cachectic patients and healthy controls [156]. Similar results were reported in an earlier Turkish study among GC patients [157]. Thus, resistin seems to have positive association with GC and/or EC, the more severe the disease, the higher the level of resistin.

Resistin and Hematologic Malignancies

Hematologic malignancies include leukemia, lymphoma, myelodysplastic syndrome and multiple myeloma. Leukemia is a group of cancers derived from bone marrow and blood. It can be classified into acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). ALL is the most common type among children and adolescents while the most common types among adults are CLL and AML [112]. Obesity is one of the prognostic factors that has been found to be associated with higher risk of relapse and poorer treatment outcome of children diagnosed with ALL. A case–control study in Greece showed children with newly diagnosed B-cell ALL had significantly higher resistin level than healthy children; in addition, the resistin level among these patients decreased at the time before the end of maintenance phase treatment, comparing to the levels at diagnosis [158]. However, no association was found between blood resistin levels and childhood ALL in a case–control study involving 54 patients and 51 controls in the UK [159]. In a study involving children receiving hematopoietic stem cell transplantation (HSCT), resistin declined within 2–3 weeks and rebounded by week 4 among children receiving HSCT treatment compared to healthy controls, suggesting that children with HSCT treatment may have predictive pattern of changes in resistin and other cytokine activities that are associated with treatment complications. Among adults, resistin level was found to be higher in lymphoma patients than in CLL, acute leukemia and healthy controls, it also correlated with the platelet count in patients with hematologic malignancies [160]. In contrast, Dalamaga et al. reported lower resistin level was associated with higher risk of non-leukemia hematologic

malignancies including myelodysplastic syndrome [161] and multiple myeloma [162], suggesting a compensatory role of resistin in response to other upregulated cytokines involved in these hematologic malignancies [14]. In another myeloma study conducted in Norway, no significant difference of resistin levels was observed between multiple myeloma patients and healthy controls [163]. Inconsistent results across these studies may be due to the differences in statistical methodologies, study design, and study population with various ethnicity.

Resistin and Renal Cancer

Renal (kidney) cancer is more common among men than women, with incidence rates being stable from 2007–2011. Results from a case–control study nested in the cohort of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study showed neither resistin nor leptin was associated with renal cell carcinoma (RCC) among smokers [164]. This study, however, found adiponectin was associated with RCC, suggesting that resistin may not be a mediating biomarker in the association between obesity and RCC.

Resistin and Pancreatic Cancer

Pancreatic cancer (PanC) is often diagnosed at late stage resulting in a low survival rate. The 1-year and 5-year survival rates combining all stage PanC are 28 % and 7 %, respectively [112]. Obesity and intrapancreatic fatty infiltration have been associated with increased PanC risk [165] and it has been postulated that obesity induces a chronic inflammatory state through adipokines and cytokines that potentially promote PanC development. As one of the adipokines and inflammation-related biomarkers, resistin expression in pancreatic islet cells of type 2 diabetic patients was much higher than that in controls [166]. A PanC-patients-only study comparing resistin level in pancreatectomy tissue in PacC patients with or without diabetes by immunohistochemical staining showed that resistin expression and colocalization with insulin were higher in the pancreas of patients with diabetes [166]. In a cross-sectional study involving 45 PanC patients, Jiang et al. found resistin expression in tumor was significantly associated with the aggressiveness and higher stage of PanC, and patients with resistin expression in tumors had worse survival than patients without resistin expression in tumors [167]. A case–control study conducted by Gasiorowska et al. showed fasting plasma resistin was much higher in PanC patients than in healthy controls, but there were no differences in plasma resistin levels between PanC patients with or without diabetes. This study did not show a correlation between resistin and other clinical characteristics such as jaundice, pain, tumor size, and metastases [168]. In sum, resistin level is associated with PanC and its aggressiveness. Insulin resistance pathway is part of the biological mechanisms that mediate this association.

Resistin and Carcinogenesis Mechanisms

Tumor progression and metastasis represent a complex pathological process. Accumulating evidence suggests that resistin may exert neoplastic effects via two mechanisms. First, it can act directly on cancer cells by stimulating proliferation and migration, promoting cell adhesion, and altering the tumor microenvironment. Second, resistin may act indirectly by modulating insulin sensitivity and glucose metabolism, regulating inflammatory responses and influencing tumor angiogenesis [14] (Fig. 9.3).

Direct Mechanisms of Action

The roles of resistin in cancer-related biomarkers and mechanisms have been explored in cultured cancer cells and animal models. In certain cancer cells, supra-physiological levels of resistin appear to repress cancer cell growth. In MDA-MB-231 human breast cancer cell line, 5–10,000 ng/ml resistin-13-peptide, which contains 13 amino acids (from 22 to 34 of human resistin molecule), decreased the activity

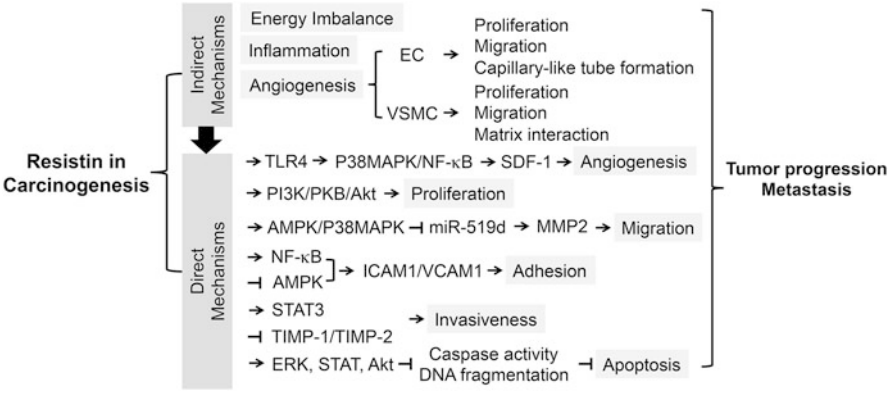

Fig. 9.3 Integrative view of mechanisms by which resistin contributes to carcinogenesis. Tumor progression and metastasis represent complex pathological processes. Accumulating evidence suggests that resistin may exert neoplastic effects via two mechanisms: (1) resistin targets directly on cancer cells by stimulating proliferation, migration, promoting cell adhesion, and altering the tumor microenvironment; (2) resistin may act indirectly by modulating glucose homeostasis and insulin resistance, regulating inflammatory responses and influencing tumor angiogenesis. *AMPK* AMP-activated protein kinase, *EC* endothelial cells, *ERK* extracellular signal-regulated kinase, *ICAM* intercellular adhesion molecule, *MAPK* mitogen-activated protein kinase, *MMP* matrix metalloproteinase, *NF-kB* nuclear factor-kappaB, *PI3K* phosphatidylinositol-3-kinase, *PKB* protein kinase B, *SDF-1* Stromal cell-derived factor-1, *STAT* signal transducer and activator of transcription, *TIMP* tissue inhibitors of metalloproteinases, *TLR4* toll-like receptor 4, *VCAM* vascular cell adhesion molecule, *VSMC* vascular smooth muscle cell. "↑" indicates stimulation and T" indicates inhibition

of matrix metalloproteinases (MMP)-2 and MMP-9 [169], family members of zinc-dependent protease involved in migration and metastasis of cancer cells due to extracellular matrix degradation [170]. Resistin also enhanced the protein expression of tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-2, and dose-dependently inhibited MDA-MB-231 cell growth and colony formation [169]. The effects were more profound at supraphysiological concentration (500 ng/ml, 5,000 ng/ml, and 10,000 ng/ml) [169]. Moreover, 2.5 mg/kg and 5 mg/kg resistin-13-peptide treatment repressed tumor growth in 5- to 6-week-old female athymic nude mice [169], although the serum concentration of resistin following treatment was not examined and reported.

Majority of the reported studies suggested that resistin enhanced cancer cell growth. Resistin (5–50 ng/ml) enhanced adhesion of SK-Hep1, a hepatoma cell line, to endothelial cells through transcription factor NF-kB activation as well as ICAM-1 and VCAM-1 expressions and inhibited endothelial AMPK activation, providing a notion that resistin may promote hepatocellular carcinoma metastasis [171]. The effects of resistin on SK-Hep1 cells were attenuated by treatment of 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) that induces AMPK activation [171]. In addition, resistin expression was higher in chondrosarcoma biopsy tissues than in normal cartilage [172]. Resistin (0.3–3 ng/ml) increased MMP-2 expression and promoted cell migration of human chondrosarcoma cells through activation of the AMPK/p38MAPK signaling pathway and downregulation of miR-519d expression [172]. In human choriocarcinoma cells (BeWo), resistin (10–100 ng/ml) enhanced MMP-2 expression, suppressed TIMP-1 and TIMP-2 and increased trophoblast-like cell invasiveness [173]. Among human prostate cancer cell lines PC-3 and DU-145, expression of resistin was also observed [66]. Treatment of resistin mature peptide (10–200 ng/ml) or overexpression of full-length resistin genes stimulated prostate cancer cell proliferation through PI3K/PKB/Akt signaling pathways [66]. In the carcinogenesis of human gastric cancer, stromal cell-derived factor-1 (SDF-1) (CXC chemokine ligand-12)/CXC chemokine receptor 4 (CXCR4) was involved, where it stimulated angiogenesis and favored metastasis of tumor cells to distant organs. In human gastric cancer cells, resistin induced expression of SDF-1 mediated by TLR4 and p38MAPK/NF-kB pathway [174]. Resistin promoted growth and aggressiveness of breast cancer cells, and these effects were mediated through signal transducer and activator of transcription 3 (STAT3) activation [175]. In HCT-116 colorectal cancer cell line, resistin mRNA and protein was not expressed in either cell lysate or supernatant, but colorectal cancer cells proliferation was induced by resistin after 24 and 48 h in a dose-dependent manner, suggesting resistin exerts biological functions in a paracrine rather than an autocrine manner [176]. Resistin (1–10 ng/ml) inhibited caspase activity and DNA fragmentation of porcine ovarian cells by activation of the ERK, STAT, and Akt signaling pathways, therefore acting as an anti-apoptotic factor [177]. Tumor-associated dendritic cells (TADCs) secreted resistin [67]. The condition medium of TADCs increased cell migration and invasion, as well as the osteolytic bone metastatic properties of lung cancer cells [67]. Neutralization of resistin of TADC condition medium abolished its malignancy-inducing features [67]. Circulating levels of

resistin was elevated in mice transplanted with lung cancer cells, tumor-infiltrating CD11c-positive DCs in human lung cancer samples and lung cancer patients' serum [67]. Mice that received anti-resistin antibodies showed a decreased incidence of cancer development and metastasis. These findings suggest that TADC-derived resistin may be a novel candidate in promoting the development of lung cancer [67].

Thus, the biological activity of resistin may be cell line-specific and dose-dependent; and truncated resistin and full-length resistin may exert distinct effects. Although resistin may function through distinct signaling pathways in different cancer-cell types, common mechanisms seem to involve proliferation, migration, adhesion, invasiveness, and angiogenesis that favor carcinogenesis and metastasis.

Indirect Mechanisms of Action

An energy imbalance underlies many human metabolic diseases, including obesity and cancer. Resistin may indirectly mediate cancer development through its metabolic effects. B16BL6 melanoma cells metastasized more aggressively in obese *ob*/*ob* than in lean mice [178]. Serum of *ob*/*ob* leptin-deficient obese mice had higher levels of resistin, insulin, tissue plasminogen activator inhibitor-1 (tPAI1), IL-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) compared to control serum, and increased the invasive ability of B16BL6 melanomas [178]. Although the effects of *ob*/*ob* mice serum on B15BL6 melanoma may not be specific to resistin, the results suggested that serum of obese mice possesses potential ontological effects, linking metabolic disorders to carcinogenesis.

Resistin regulates ovary carcinoma production of vascular endothelial growth factor (VEGF) and the angiogenic processes. In human ovarian epithelial carcinoma cells (HO-8910), resistin (10–150 ng/ml) enhanced vascular endothelial growth factor (VEGF) expression in a time- and concentration-dependent manner [179]. Furthermore, resistin enhanced DNA-binding activity of Sp1 with VEGF promoter in a PI3K/Akt-dependent manner, resulting in the upregulation of VEGF expression [179]. In an in vitro angiogenesis system for endothelial cells (EA.hy926) cocultured with HO-8910 cells, the addition of resistin stimulated endothelial cell tube formation, which was abolished by VEGF neutralizing antibody [179]. Resistin induced endothelial proliferation, migration and capillary-like tube formation of human coronary artery endothelial cells (HCAECs). Both resistin-induced HCAEAs proliferation and migration were effectively blocked by a resistin-neutralization antibody. Resistin also significantly upregulated the mRNA expression of vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2), MMP-1 and MMP-2, and induced phosphorylation of extracellular signal-regulated kinases 1/2 (ERK1/2) and p38MAPK [180]. In addition, in endothelial EA.hy 926 cells, resistin increased the expression of MCP-1 and platelet endothelial cell adhesion molecule (PECAM-1) as well as adhesion of monocytes to endothelial cells [181]. Resistin promoted human aortic smooth muscle cell

(HASMCs) proliferation through activation of ERK1/2 and PI3K pathways [86], and stimulated matrix interaction and migration [87], both important processes in metastasis and angiogenesis.

Resistin in Cardiotoxicity of Chemotherapy

Resistin has been associated with risk of atherosclerosis [81] and heart failure [182, 183], mainly attributable to its role in regulating inflammation and glucose homeostasis. The recent large-scale clinical cohorts have connected elevated serum resistin levels with the subsequent development of heart failure [182, 183]. Indeed, mouse and human resistin impair glucose transport in primary mouse cardiomyocytes [26]. Resistin overexpression altered cardiac contractility and promoted cardiac hypertrophy possibly via the IRS-1/MAPK pathway [184].

Anthracycline-induced cardiotoxicity has long been recognized as a common and serious adverse effect of treatment for many malignancies [185]. Serum resistin levels in women undergoing anthracycline-containing chemotherapy increased significantly at 3 months and remained elevated at 6 months in those with subsequent cardiotoxicity [186]. Further, elevation in resistin correlated with decline in ejection fraction in these women, suggesting the potential role of resistin in acute anthracycline-induced cardiotoxicity [186]. Indeed, in a humanized mouse model lacking murine resistin but transgenic for the human resistin gene (BAC-Retn) [38], doxorubicin led to a 4-fold induction of serum resistin levels in BAC-Retn mice. Doxorubicin-induced cardiotoxicity was greater in the BAC-Retn mice than in littermate controls not expressing human resistin (Retn knockout), with increased cardiac mRNA levels of inflammatory and cell adhesion genes [186]. Thus, the elevated resistin may be a biomarker of anthracycline-induced cardiotoxicity [186] and resistin may represent a therapeutic target for chemotherapy-induced cardiac toxicity as well as cardiovascular diseases.

Conclusion and Perspectives

Obesity contributes to multiple comorbidities, such as diabetes, cardiovascular diseases, and cancers, likely via shared mechanisms involving insulin resistance, inflammation, and oxidative stress [18, 187]. A number of adipose-derived factors mediate energy balance, metabolism, and inflammatory responses, and are recognized as major contributors to the diverse consequences of obesity [10–12]. Resistin is emerging as a key biomarker in understanding the complexity of these obesity-related biological processes due to its central role in promoting insulin resistance and inflammation [4–7], both are essential pathways linking obesity to the increased risk of cancer [13–15].

Unlike other adipokines, resistin shows a distinct expression pattern between rodents and human as resistin is expressed exclusively by adipocyte in rodents, while mainly by monocytes and macrophages in human. It is therefore critical to recognize the limitations of translating results derived from rodents to human subjects [5]. The establishment of humanized resistin mice has provided a useful model to delineate the role of human resistin in an in vivo setting. CAP1, a resistin receptor with 97 % homology between human and rodents, has recently been identified [36]. As the role of CAP1-mediated signaling has only been examined in human THP-1 monocyte cell lines [36], the downstream signaling pathways of CAP1 in other cell and organ systems require further investigation. It is also essential to map out resistin receptor-dependent and independent signaling pathways in obesity and its related comorbidities.

Although epidemiological studies generally support the linkage between higher resistin levels and increased risk of certain types of cancer, especially breast cancer, endometrial cancer, colorectal cancer, lung cancer, gastric and esophageal cancer and acute lymphoblastic leukemia, this association remains undetermined for many other types of cancer. For example, there have been no epidemiological studies on resistin and ovarian cancer, although resistin levels were elevated in women with polycystic ovary syndrome, a common endocrine disorder associated with insulin resistance and female cancers [188, 189]. The association between obesity and increased risk of hepatocellular carcinoma (HCC), which comprises most primary liver cancer, was well established [190], but there is a lack of human epidemiological evidence for the association between resistin and HCC risk. In addition, there have been no studies examining the correlations between resistin and molecular subtypes of cancer, critical predictors of cancer mortality. Moreover, most of the reported epidemiological studies were hospital-based case–control studies, which have limited generalizability of results, and may have selection bias due to uncontrolled factors that lead the hospital cases and controls systematically different from each other. In addition, the studies with negative findings may not have enough power to detect the possible associations due to limited sample size. Future case–control studies with adequate sampling procedure and larger sample size are needed. Prospective studies should also be implemented to explore the ontological role of resistin in cancer promotion and progression. In addition, measurement of urine or saliva resistin levels may be incorporated to explore their correlation with serum resistin levels and associations with cancer risk, because of the easiness of sample collection suitable for large population-based epidemiological studies. Last but not the least, recent meta-analysis has suggested the association between circulating resistin levels with cardiovascular and all-cause mortality in high-risk patients with chronic diseases [191], but no studies have examined resistin levels with cancer-specific mortality. As cancer is the second leading cause of mortality in the USA, it is imperative to determine whether resistin acts as a risk factor for cancer-specific mortality.

It is important to acknowledge that, compared with other adipokines, such as adiponectin and leptin, the role of resistin in obesity-associated cancer risk has been less heavily investigated. The relative contribution of resistin versus other adipose-derived factors should be further evaluated to gain integrative insights into the

impact of adipose tissue-derived factors on obesity-related malignancies. Nonetheless, the additive or synergistic effects of obesity-related dysregulation of multiple adipokines might represent a more complete picture for the link between obesity and cancer.

Despite progresses being summarized in this chapter, we are still in the early phases of exploring the complex biological and epidemiological characteristics of resistin. Further research is needed in the following areas to: (1) investigate the mechanisms for the regulation of human resistin expression, receptor-dependent and independent signaling pathways in obesity-related cancer promotion and progression; (2) develop reliable and standardized laboratory techniques to evaluate total resistin levels and its isoforms in multiple sources of biological specimen in both basic research and clinical laboratories; (3) with prospective studies, explore the utilization of resistin levels, either alone or in conjunction with other adipose-derived factors, as a diagnostic and prognostic biomarker of obesity-related carcinogenesis; (4) with large scale GWASs, determine whether resistin gene polymorphisms are causally associated with cancer incidence and prognosis; (5) assess the association of resistin level with molecular subtypes of cancer and cancer-specific mortality; (6) evaluate whether neutralization of resistin or inhibition of its expression and downstream signaling may provide therapeutic implication.

In summary, the central biological role of resistin in inflammation and insulin resistance, higher expression of resistin in cancerous tissue, and clear evidence of resistin in promoting proliferation, migration, adhesion, angiogenesis and invasiveness of cancer cell lines, are suggesting that resistin plays important roles in cancer promotion, progression and metastasis. Resistin may not only act as a potential biomarker for cancer development and progression, but also represent an inflammatory and metabolic modulator linking obesity to cancer. Despite inconsistencies in the literature and the need for additional biochemical, genetic, and prospective studies, resistin provides a biological framework for understanding the interrelations between obesity and obesity-associated malignancies.

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Chapter 10
Role of C-Reactive Protein in Cancer

Helen Swede and Dejana Braithwaite

Abstract Low-grade chronic inflammation is a proposed mechanism linking excess body weight with cancer development. Adipocytes, particularly visceral fat cells, regularly release pro-inflammatory cytokines likely due to hypoxic and necrotic conditions in conglomerated fat tissue in obese individuals. C-reactive protein (CRP), a long-established acute-phase reactant, is triggered primarily by the cytokine Interleukin-6, as part of the innate immune system apparatus to resolve acute infection, injury, and inflammation. Some but not all epidemiologic studies suggest that low-grade circulating CRP levels predict cancer development, particularly for lung and colon cancers. Evidence as a prognostic factor, however, is more convincing, but whether or not elevated CRP reflects inflammation associated with large, aggressive neoplasms or solely serves as a general marker of mortality risk needs clarification.

Keywords C-reactive protein • Obesity • Adipocytokines • Acute phase reactants • Cancer • Cancer etiology • Cancer prognosis • Malignant neoplasms • Chronic inflammation • Innate immune system

Overview

C-reactive protein (CRP), an acute phase-reactant detectable in peripheral blood, is used clinically as a sensitive yet nonspecific marker of inflammation. Established uses are to detect and monitor acute infections, disease activity in certain chronic immunologic disorders, and response to anti-inflammatory medications [1–3].

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Additionally, while cardiology practice guidelines suggest that clinicians may consider assessment of CRP level in asymptomatic patients at intermediate risk for cardiovascular (CVD) events [4], consensus is not yet in place [5]. The U.S. Preventive Services Task Force has concluded that, despite strong evidence that CRP is associated with CVD events, data that reducing CRP level prevents these outcomes are lacking [6]. In recent years, cancer researchers have focused on elevated CRP as a marker of chronic low-grade inflammation, which, as acquired by various behavioral risk factors and characterized by a number of different underlying mechanisms, can promote cancer development and progression [3, 7]. There is long-standing consensus that cancer risk at certain sites is due directly to chronic infection. Hepatitis B virus, human papilloma virus, or H. pylori, for example, are virtually obligate causes for, respectively, liver, cervical, and gastric cancer [8–10]. Additional evidence supporting a carcinogenic role of chronic inflammation stems from the association of ulcerative colitis (UC) and, to a lesser extent, Crohn's disease with increased risk for colon cancer [11, 12]. Lastly, dietary intake, physical inactivity, and metabolic disturbances such as diabetes or insulin resistance have been linked to increased cancer risk through pro-inflammatory processes [13]. There remains continuing debate, however, if elevated CRP has a direct role in carcinogenesis or is simply a reflection of recruitment and activation of immune cells at the site of a growing neoplasm impinging upon surrounding tissues as well as a response to tumor antigens [14–16].

CRP Function

Inflammation is commonly observed as part of the defense apparatus triggered to protect the host from pathogens [11]. Elevated CRP is one of a multitude of physiologic responses to signals generated at a site of injury, irritation, or infection [1, 3, 7]. It was first described in 1930 by Tillet and Francis who observed that serum of febrile patients in the midst of the acute phase of pneumonia precipitated in contact with soluble extract of C-polysaccharide components of *Streptococcus pneumonia* bacteria, suggesting that a primary function of CRP is clearance of encapsulated bacteria [17]. Moreover, the reaction did not occur after the pneumonia was resolved, and, in later studies, was found to occur in extracts from other bacteria and fungi but not in the presence of a viral infection [3]. While the first discoveries of these reactions occurred during acute phases of an infection, elevated levels of CRP and other reactants now have been linked to numerous immunologically mediated chronic diseases, physical trauma, postsurgical irritation, psychological stress, childbirth, and advanced neoplasia [1, 18]. CRP is produced by hepatocytes in response to Interleukin-6 (IL-6), primarily, and other cytokines secreted by T-cells and macrophages at the local site [19]. Accumulating evidence also has demonstrated production of CRP by adipocytes, particularly in the visceral fat [20], which represents a proposed pro-inflammatory mechanism linking obesity and cancer risk as discussed in more detail below.

Starting in the early phase of an active infection, CRP plays an integral role in the innate host defense system through its recognition of and binding to microorganisms and then recruiting the complement system and phagocytic cells [21]. The protein also directly eliminates apoptotic and necrotic host cells [22], thus restoring normal structure and function to injured tissues. In response to a stimulus (e.g., IL-6), CRP production is synthesized within 1–2 days to concentrations 500–1000 times greater than basal [23]. With a half-life of about 19 h [24], CRP levels can be expected to decrease comparatively quickly after the acute stimulus is gone or when there is restoration of tissue structure and function [23]. Hence, elevated CRP also can be present in the context of sustained tissue inflammation outside the context of acute infection, and it is this low-grade state upon which cancer research is focused. IL-6 and other acute-phase cytokines typically have a shorter half-life of 2–6 h making these markers less stable markers of current inflammation. Other strengths of CRP as a clinical and research tool is that it appears to lack seasonal or diurnal variation [25], is stable in long-term storage at ≤ −20 °C [26] and inexpensive, highly sensitive assays are readily available [6].

Obesity and Elevated CRP

A key determinant of moderately elevated CRP is obesity (BMI > 30.0), particularly visceral fat stores [27]. Until recently, adipose cells were thought to function as inert storage receptacles of excess energy [28] [20]. It now has been shown definitively that adipocytes generate a state of chronic low-grade inflammation as demonstrated by the strong and consistent correlation with increased markers of inflammation and macrophages [20]. As stated, adipocytes, particularly visceral fat cells, are a significant source of extrahepatic CRP production primarily as a consequence of the secretion of IL-6 by fat cells (Khaodhiar et al. 2004, [20]).

What causes adipocytes to secrete pro-inflammatory cytokines and acute phase proteins? Some postulate that hypoxic and necrotic segments in the fat tissue of obese individuals might trigger activation of inflammatory macrophages (Arcidiacono et al. 2012; Aron-Wisnewsky et al. 2012). Additionally, an inflammatory response is thought to be generated when excess caloric intake results in larger adipocytes which then initiate local cellular stress responses [27]. Further, there is evidence of coordination between macrophages/adipocytes, in white fat tissue, with cells of the immune system (Arcidiacono et al. 2012). White adipose tissue provides a long-term fuel reserve for the organism whereas brown adipose tissue plays a major role in heat production (Cannon and Nedergaard 2004). Numerous epidemiologic reports have shown strong correlations of elevated CRP with visceral obesity as characterized by waist circumference (WC), on the order of $r = 0.40–0.61$ [27]. Nonetheless, there remains strong evidence that obesity as measured by BMI is associated with CRP elevation. In a recent study using the NHANES III dataset ($n = 7072$, age ≥ 50), elevated CRP was positively correlated with BMI ($p = <0.0001$): 37.9 % ($n = 705$) of the obese (BMI > 30.0) exhibited levels

of CRP indicative of chronic low-grade inflammation compared to 30.0 % ($n = 831$) of the overweight (BMI 25.0–29.9) and 22.1 % ($n = 506$) of the normal weight participants (BMI > 18.5–29.9) [16]. In another report, BMI explained 5–15 % of plasma CRP variation in a large meta-analysis consisting of over 80,000 participants from 15 major study cohorts of persons with European ancestry [29].

Genetic Basis of CRP Level

The gene encoding CRP is located on the long arm of chromosome 1 located between 1q21 and 1q23 (Whitehead et al. 1983). Twin studies have estimated that approximately one-half of the variation in baseline CRP level can be attributed to inborn traits (MacGregor et al. 2004). In family studies, CRP concentrations were shown to be 35–40 % heritable [30]. While approximately 20 CRP or CRP-related polymorphisms have been identified and linked with variation in basal level [15, 31–33], associations with disease outcomes including cancer have been inconsistent. Interestingly, Greenfield et al. [34] reported in a study of monozygotic twins that obesity is a key determinant of baseline CRP level independent of genetic status [34]. To date, the vast majority of genetic studies of CRP have been in the field of cardiovascular and autoimmune diseases. It remains unknown if the influence of genetic polymorphisms is relevant to cancer and for which particular sites, and, if gene–environment interactions play an important role.

There are multiple and complex genetic influences of CRP expression. Functional variants directly affect the transcription or the stability transcript whereas nonfunctional variants appear to be in linkage disequilibrium with the functional polymorphisms [14]. Notably, genetic variation at the CRP locus explains a fraction of variation in baseline CRP levels. Another group of genetic variants, referred to as trans-acting variants (i.e., remote genes which indirectly affect CRP levels), also are known to play a crucial role in the regulation of CRP expression [14]. Due to these multiple genetic influences as well as the possibility of reverse causality (i.e., elevated CRP levels resulting from occult cancer), it has been recommended that epidemiologic studies investigating a causal role in cancer development use a Mendelian randomization design. Allin and Nordestgaard [14] provide a thorough discussion of this approach. In brief, in conventional observational studies, it is nearly impossible to distinguish elevated CRP due to the presence of subclinical neoplasms from elevated CRP playing etiologic role. Also, due to lack of random assignment to exposure arms, observational studies are prone to bias from unequal distribution of potential confounders across study groups. Mendelian randomization allows a direct assessment of cancer risk, however, by examination of genetic variants known to influence basal CRP level, and under the assumption of random assortment of alleles from parents to offspring as well as the obvious independence between

genotypes and behavioral risk factors (e.g., smoking), equivalent distribution of known and unknown potential confounders in genotype groups can be assured. Therefore, a higher cancer risk in the group carrying alleles that increase CRP levels compared to the group with alleles that decrease CRP levels would provide an unbiased estimate of causality. For comparative analyses, Mendelian randomization studies also typically report correlations of CRP level with both genotype and cancer risk.

Population Prevalence of CRP Levels

Basal CRP level can have an interindividual range of one-hundred-fold in the population due to behavioral risk factors, presence of certain chronic diseases, and constitutional genetics [14, 35]. Conventional CRP tests have a sensitivity of >2.0 mg/L [16] and typically are used to measure disease activity during a clinically defined infection or major inflammatory diseases [14]. The more recently developed high-sensitivity CRP test (hs-CRP) has a lower level of detection of 0.2 mg/L which allows allowing more precise quantification of low-grade inflammatory states [7, 36]. Another benefit of the hs-CRP assay is that the range of values favors normality and is wide, thereby reducing the necessity for data transformation or imputation. Basal circulating level of CRP in most healthy subjects is usually around <1 mg/L, and, by convention, >10 mg/L is the cut-point for acute infection, rheumatoid arthritis, and advanced cancer [14, 23]. In cardiology research and practice, a CRP level of >1 mg/L to <10.0 mg/L is considered indicative of disease risk associated with chronic low-grade inflammation [7], though others have employed cut-points of 2 mg/L [2] or 3 mg/L [6, 25, 36]. In cancer research, ranges for clinically meaningful risk categories of hs-CRP elevation have not yet been defined but risk is often assessed using the cut-points established in cardiology studies.

Understanding prevalence and epidemiological variation in elevated CRP level has relevance to designing epidemiologic studies, incorporation of potential confounders into multivariate models, and estimating attributable risk. A recent surveillance study by Ong et al. [36] of the distribution of hs-CRP values in the population using NHANES continuous datasets (1999–2010) reported that persons with elevated hs-CRP were more likely to be women, less educated, cigarette smokers, non-Hispanic Black or Mexican-American (all adj. $P < 0.01$) [36]. Similar differences were observed in studies of the earlier NHANES III dataset that used the conventional CRP assay [37, 38], and in clinical cohort studies such as the Dallas Heart Study ($n = 2749$, age 30–65) [39] and Health, Aging, and Body Composition Study ($n = 2490$, age 70–79) [40]. Ong *et al.* [36] also observed that the prevalence of elevated hs-CRP (i.e., >3.0 mg/L) decreased somewhat during the study period from 36.7 % in 1999–2002 to 32.0 % in 2007–2010 (Adj. $P < 0.001$), which corresponded to mean levels of 1.92–1.66 mg/L

Table 10.1 Non-acute sources of variability associated with elevated or reduced circulating CRP levels

Elevated levels
Obesity (BMI ≥ 30.0)
Excess abdominal adiposity
Advancing age
Ulcerative colitis
Crohn's disease
Diverticular diseases
High blood pressure
Cigarette smoking
Diabetes
Metabolic syndrome/insulin resistance
Low HDL/high triglycerides
Fibrinogen
Estrogen/progesterone use
Gingivitis
Periodontal disease
Chronic kidney disease and failure
Chronic bronchitis and other respiratory disorders
Rheumatoid arthritis and other inflammatory disorders
Advanced neoplasms
Male
Reduced levels
Moderate alcohol intake
Physical activity/exercise
Weight loss
Statins
Niacin

(Adj. $P < 0.001$) [36]. Reason for the decrease over time is unclear but authors hypothesized that the downward trend might be due to greater use of statin drugs and concomitant reduction in the pro-inflammatory milieu. Interestingly, however, CRP levels also declined, but to a lesser degree, among participants who did not report current statin use. Authors noted that a number of other NHANES studies during this time period reported better clinical control of other pro-inflammatory conditions such as diabetes, hypertension, and hypercholesterolemia in the US population [41–46]. Table 10.1 contains a list of non-acute factors that can raise or lower CRP level, important considerations when designing and analyzing clinical studies.

Cancer Risk and Elevated CRP

A number of large prospective epidemiologic studies have reported that elevated circulating CRP level predicted cancer incidence in presumably healthy individuals at baseline [14, 35] but the extant evidence remains largely inconsistent. Below is an overview of major studies examining a causal link with overall cancer risk and several top cancer sites. A large Danish prospective cohort analysis ($n = 10{,}408$) published in 2009 reported a significant 1.3-fold increased risk for **any cancer** when comparing highest and lowest quintiles of CRP [47]. This study also found a twofold increased risk of **lung cancer**, which, conceivably, could be the main driver for the significant finding for overall cancer given its high incidence in the population. A nested case–control study ($n = 592$ cases) also found an association with **lung cancer** when comparing highest to lowest quartiles of CRP level [48]. A recent meta-analysis of lung cancer studies (10 studies, n = 1918 cases) reported a pooled relative risk of 1.18 (95 % CI 1.09–1.28) for lung cancer in males per each logarithmic unit change in CRP level but this effect was not observed in females [49]. Nor was an association observed between IL-6 and lung cancer. A subsequent nested case–control analysis by Sheils et al. [50] of 526 patients with lung cancer and 592 healthy controls, reported an elevated Odds Ratio of 2.27 (95 % CI 1.51–3.41) when comparing the highest to lowest CRP group, which did not change appreciably when accounting for reverse causation by excluding incident lung cancer cases occurring two years or less after entry into the study. Two recent review papers discuss strong clinical and epidemiologic evidence of a link between chronic infection and inflammation with lung cancer risk [51] including a possible pathogenic role of elevated CRP [52]. Given that a small minority of smokers develop lung cancer (<15 %), the link with chronic inflammation is a thought-provoking possible interaction.

Of the major prospective cohorts that assessed the link between baseline CRP and **colorectal cancer** development, the majority reported positive associations [16, 40, 53–57] while some did not [58, 59]. Relative risks in the positive studies ranged from 1.4 to 2.9 using different CRP categorization schemes (e.g., quartiles, clinical cutpoints.) Notably, Izano et al. [40] was the first to analyze longitudinal CRP measurements (i.e., 4 time points in 8 years), and found a substantial effect (2.29; 95 % CI: 1.08–4.86) for updated CRP level in a comparison of highest to lowest tertiles [40]. In a study using the NHANES III dataset ($n = 7072$), Swede et al. [16] found an interaction with BMI, whereby the strongest estimate of effect for elevated CRP (≥2.2 mg/L), compared to undetected level, was seen among normal weight participants (2.47, 95 % CI: 1.10–5.54) [16]. Moreover, null findings were observed among the overweight and obese, suggesting that, statistically, the independent influence of CRP on cancer risk might be observed more clearly outside the myriad of obesity-induced metabolic disorders (e.g., metabolic syndrome) that also are linked with development of colorectal cancer [16]. In these two studies, however, no associations were observed with other obesity-related cancers (e.g., **breast, endometrial, prostate, pancreas**). A recent meta-analysis ($n = 4{,}706$

cases) by Zhou et al. [60] reported pooled increased relative risks for colon cancer per one unit change of logarithmic transformed CRP (1.13 95 % CI 1.05–1.21) but not for rectal cancer (1.03, 95 % CI 0.90–1.17) [60]. No significant association was found between IL-6 and colorectal cancer, however.

For **breast cancer**, a meta-analysis by Chan et al. (2015) of 12 prospective studies ($n = 3{,}522$ cases among $n = 69{,}610$ women) reported that for each doubling of CRP concentration, a 7 % [95 % CI, 2–12 %] increased risk was observed; the association was linear over most of the range of CRP concentrations. Authors suggest that additional prospective analyses are needed to better understand confounding and effect modification from lifestyle factors. Another recent meta-analysis by Wang et al. [61] across 11 prospective studies ($n = 5371$ cases) also reported a modest, but statistically significant association for breast cancer when comparing highest to lowest CRP categories (pooled RR = 1.27 95 % 1.07–1.49) [61].

For **prostate cancer**, in a review of 14 etiologic studies, there appeared to be no evidence for a causal link between elevated CRP [14] which is surprising given emerging hypotheses and data that prostate cancer might have infectious and inflammatory origins and might not be exclusively a disease of aging [62].

Allin and Nordestgaard [14] conclude that evidence from Mendelian randomization studies largely indicates that elevated circulating levels of CRP do not cause cancer [14]. The first Mendelian randomization study ($n = 6000$) of the link between CRP polymorphisms and cancer risk [63] reported no evidence of a causal link with **colorectal, prostate,** and **breast cancers.** A 2.6-fold greater risk was observed for **lung cancer**, however, among individuals who harbored a rare CRP allele (rs1205); the authors of the study speculated that this finding could be explained by a high level of chronic inflammation related to carcinogens in tobacco smoke. This lung cancer finding has not been replicated in subsequent Mendelian studies [14] but associations have been observed in several observational studies as highlighted above. A large Mendelian randomization prospective study in a Danish population ($n = 8{,}224$) of four SNPs in the CRP gene locus observed a modestly increased Hazard Ratio of 1.09 (95 % CI 1.03–1.14) for risk of **any cancer** per doubling of circulating CRP level [64]. Although CRP loci were associated with up to a 72 % increase in circulating level in a cross-sectional analysis ($n = 36{,}403$), there were no causal associations between CRP genotypes and **lung, colorectal, breast, prostate, bladder, and urinary tract cancers** in the prospective arm of this study [64]. Lastly, causal inference is also hampered because while weight loss, exercise, smoking cessation, and statin drugs can reduce CRP level [2, 65, 66], it remains unknown if reduction lowers cancer risk. As stated above, current evidence in cardiology indicates that reduction of CRP does not result in fewer CVD events [6].

Prognosis and Elevated CRP

Data supporting a role of CRP in assessing prognosis is more consistent and derived from a larger evidence base than found in cancer risk studies. The adverse effect of elevated CRP on prognosis appears to be independent of disease stage. In a

systematic review of 12 studies, Pathak et al. [67] reported that overall survival and disease-free survival were shorter in the presence of elevated preoperative CRP in local and advanced **colorectal cancer.** [67] Given the limited number of high-quality studies, however, authors cautioned that there currently is insufficient evidence to justify its routine use. Similarly, in **breast cancer**, a recent meta-analysis consisting of 10 studies (n = 4,502) reported a combined Hazard Ratio of 2.08 (95 % CI, 1.48–2.94) for cancer-specific survival in relation to elevated CRP [68]. Surprisingly, elevated CRP level at diagnosis differentiated prognosis as reported in meta-analyses of **non-small cell lung** [69], **ovarian** [70], and **pancreatic cancers** even though these malignancies generally confer uniformly dismal survival. Likewise, CRP was associated with an adverse prognosis even in **metastatic prostate cancer** (i.e., meta-analysis of 6 studies and 659 patients) in both castration-sensitive and castration-resistant disease [71]. Elevated circulating CRP level was shown to confer a worse prognosis in several other solid cancers in individual studies of the following malignancies: **endometrium** [72], **cervix** [73], **hepatocellular** [74], **esophagus** [75], **muscle-invasive bladder** [76], and **renal cell** [77].

Putative Mechanisms in Cancer Etiology and Prognosis

The carcinogenic role of CRP remains unclear due to limited information about CRP activity in local tissue. In general, however, inflammation is recognized as a key factor in cancer etiology. Hanahan and Weinberg (2011), in an update to their seminal review of the six hallmarks of cancer (Hanahan and Weinberg 2000), refs..

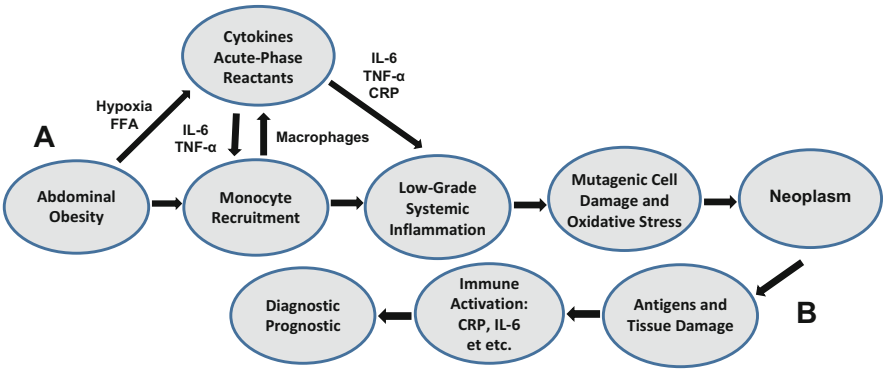

Fig. 10.1 CRC and cancer: putative pathways (**a**) Hypoxia resulting from injury to abdominal adipocytes in the obese (e.g., necrotic fat cells) can trigger release of cytokines (IL-6, TNF-α), macrophages, and acute phase reactants (CRP, primarily) leading to a chronic state of low-grade systemic inflammation. Free fatty acids (FFA) released by fat cells also can induce an immune system cascade. A possible carcinogenic mechanism could be DNA mutagenesis related to cellular damage from oxidative stress. (**b**) Neoplasms might induce an immune reaction in response to tumor antigens on cell surfaces, or, from intra- or extra-tumoral cellular injury as a result of impingement from the growing mass. Elevated circulating CRP can be indicative of advanced neoplasms; and, the degree of prognostic importance might be correlated positively with tumor size or aggressiveness

included inflammatory responses by innate immune cells as an "*enabling characteristic*." Pathologists have long observed that tumors are densely infiltrated by cells of the innate and adaptive aspects of the immune system (Dvorak, 1986). For many years, this phenomenon was thought to reflect antitumor activity, but accumulating evidence has revealed, paradoxically, that sustained inflammation promotes hyperplasia, dysplasia, and mutagenic events (Hanahan and Weinberg 2011). Chronic inflammation is thought to create a tumorigenic environment through cellular injury, resultant DNA damage, and promotion of angiogenesis [78]. Unscheduled, poorly regulated necrotic cell death also has been proposed as a molecular mechanism for inflammation-related carcinogenesis [79], which could give rise to CRP-related hypotheses due to its central role in local clearance of necrotic debris. Figure 10.1 depicts a putative carcinogenic pathway from abdominal obesity to neoplasm via elevated CRP; and, possible prognostic underpinnings of elevated CRP.

While it remains unknown if CRP has a direct local effect in increasing cancer risk, clues can be taken from Crohn's disease in which adipocytes lining the intestine from patients have been found to be a prominent locoregional source of CRP production [28]. Also, other studies have revealed CRP production in human lung epithelia in response to inflammatory stimuli [80]. Correlation of circulating CRP with local activity in cancer and normal tissue would be an important next step in characterizing a possible etiologic role [16, 56, 61]. Due to the relative ease in accessing colonic tissue through colonoscopy, colorectal cancer might serve as a general model for mechanistic studies. Microscopic pre-neoplastic colonic lesions known as aberrant crypt foci (ACF) also might provide research utility to examine a stepwise progression to cancer. There are reports linking ACF prevalence with pro-inflammatory states such as obesity [81, 82], cigarette smoking [83], and insulin-resistance [81]. For other cancer sites, assessment of CRP status in frozen samples compared to normal tissues may be feasible due to reports of CRP stability over time [26]. Methodologically, only Izano et al. [40] to date has assessed CRP level at several time points over multiple years, and longitudinal analyses might provide insight into an etiologic role of CRP [52]. Regarding a mechanism related to reduced survival, elevated CRP at diagnosis might signify extensive anatomical burden, of the neoplasm, or, given that CRP is linked to all-cause mortality primarily through cardiovascular disease—poor outcomes in cancer patients with elevated CRP simply might be confounded by a higher rate of overall mortality associated [14]. Evaluating cancer-specific cause of death using a sub-distribution competing risks analysis might clarify this issue in that all deaths are treated as informative and can provide more realistic risk estimates for the primary endpoint [84]. Nonetheless, if CRP simply reflects a state of inflammation, it might serve as a systemic alarm and perhaps other more direct targets may be effective for intervention [85].

Translational Value and Future Research Directions

While evidence of CRP as a prognostic factor in cancer is convincing, whether or not an elevated level reflects inflammation at the site of origin or serves as a general marker of mortality risk is in need of clarification. Epidemiologic evidence is limited and

inconclusive regarding a causal role of CRP, and, clearly, the body Mendelian randomization studies suggest that there might not be an etiologic influence. On the other hand, the increasing use of effective anti-inflammatory medications, such as statins, has created a methodological challenge for research because there might be fewer persons with elevated CRP—resulting a need for larger studies to achieve adequate statistical power to detect causal links. Although there is suggestive evidence of a local presence of CRP in Crohn's disease, inflamed lung epithelia, and adipocytes, there remains a paucity of data on the correlation between circulating CRP and inflammatory activity in local tissue. Further, mechanisms regulating CRP synthesis in extrahepatic sources are largely unknown. Establishing a more specific utility of circulating CRP level could aid assessment of cancer prevention interventions, development of targeted anticancer therapy, and monitoring for recurrence after a diagnosis of cancer.

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Chapter 11
GI Peptides, Energy Balance, and Cancer

Debora S. Bruno and M. Michael Wolfe

Abstract Beyond its role in nutrient absorption, the gastrointestinal tract is a vital endocrine organ responsible for the production of a variety of regulatory peptides that modulate energy balance. In this chapter, the most studied gastrointestinal peptides are described, with emphasis on their roles played in appetite control, glucose homeostasis, and weight disorders. Translational research investigating drug development targeting the "gut–brain" axis regulatory pathway for the treatment of obesity and diabetes is also highlighted. Several GI peptides have proliferative and/or pro-apoptotic functions and their possible cancer-related effects are discussed.

Keywords GI regulatory peptides • Glucagon-like peptides • Ghrelin • Incretins • Gastric inhibitory protein • Vasoactive intestinal peptide • Cholecystokinin • Polypeptide Y • Gastrin

Introduction

Obesity is a global public health issue currently affecting almost 40% of adult men and women worldwide [1]. Being associated with an increased risk for development of several malignancies, obesity will soon surpass tobacco as the leading cause of preventable cancers in the USA and worldwide [2]. The metabolic syndrome, defined by the presence of central obesity, hypertension, dyslipidemia, nonalcoholic fatty liver disease (NAFLD), and insulin resistance, is a common complication [3].

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The presence of metabolic syndrome commonly precedes the development of Type 2 diabetes mellitus (T2DM) and cardiovascular disease [4].

The hypothalamus, which is the primary output node for the limbic system, contains a number of nuclei that are responsible for controlling endocrine, autonomic, and behavioral functions. In regulating appetite and food intake, hypothalamic nuclei, such as the arcuate nucleus (ARC) and the paraventricular, the ventromedial, and the dorsomedial nuclei, are all interconnected as part of an energy homeostasis regulating circuit [5].

Amino-acid transmitters—also called neuropeptides—synthesized in the hypothalamus, brainstem and the intestine by neuroendocrine cells mediate the so-called "gut–brain axis" regulatory pathway. The concept of the gastrointestinal (GI) tract as an endocrine system was actually first coined in 1973, when cholecystokinin (CKK) was reported to decrease food intake in rats [6]. Most recently, an ever expanding list of gastrointestinal neuropeptides have been identified that stimulate the release of neuropeptides within the hypothalamic and brainstem thereby playing a physiological role in the control of feeding behaviors and ultimately of energy balance (see Table 11.1).

Roux-en-Y gastric bypass (RYBG) surgery has been proven the most effective therapy to date for treating morbid obesity. RYGB not only induces sustained weight loss but also leads to improved glucose homeostasis and insulin sensitivity [7]. A meta-analysis of more than 20,000 cases demonstrated that RYGB led to a mean of 61.2% of excess weight loss and complete resolution of diabetes in 76.8% of patients [8]. The mechanisms behind the effectiveness of the procedure are still not completely understood. Although caloric restriction may play a role in the initial weight loss that follows surgery, other mechanisms may be responsible for the long-term sustained benefits [9]. For instance, there have been documented changes in the levels of some GI regulatory peptides involved in the control of energy balance and glucose homeostasis [10], such as glucagon-like peptide-1 (GLP-1), GIP, peptide YY (PYY), and ghrelin [11–13] (see Table 11.1).

In this review, we describe the physiological roles attributed to the gastrointestinal peptides mostly studied in relation to energy balance, focusing on translational research investigating their role in metabolism and cancer biology (see Table 11.2).

Ghrelin: The Hunger Hormone

Ghrelin is the only established orexigenic hormone to have been identified to date. While a whole range of regulatory peptides responsible for appetite modulation act in a paracrine manner, being released and acting directly in the hypothalamus, ghrelin is released mostly from the GI tract (see Fig. 11.1). Ghrelin is an endogenous ligand for the growth hormone secretagogue receptors (GHSRs) [14, 15] located in the anterior pituitary gland and hypothalamus [16], among other tissues. An acylated 28-amino acid peptide, it is synthesized throughout the GI tract, with the highest levels of peptide production detected within X/A-like cells in the gastric fundus [17, 18]. A limited region in the arcuate nucleus of the hypothalamus also contains ghrelin-producing neurons [14]. The peptide can, however, be detected in the circulation of healthy humans [19].

Table 11.1 Gastrointestinal regulatory peptides: effects on energy balance, glucose homeostasis, and changes in circulating levels after gastric bypass surgery

Peptide	Effects on appetite	Effects on body weight and adipose mass	Effects on glucose homeostasis	Effects of RYGB surgery on peptide levels
Ghrelin	Orexigenic ("hunger hormone")	Plasma levels correlate inversely with BMI	Impairs insulin secretion	Circulating levels decreased in some studies (unchanged in others)
Gastric inhibitory polypeptide (GIP)	No effect	Promotes the expansion of adipose tissue	Promotes insulin secretion	Postprandial circulating levels decrease
Glucagon-like peptide-1 (GLP-1)	Anorexigenic	Induces weight loss	Promotes insulin secretion and pancreatic islet β-cell mass expansion	Postprandial circulating levels increase
Vasoactive intestinal peptide (VIP)	Levels are abnormally increased in anorexic individuals and decreased on obese subjects	May play a role in promoting weight gain. VIP-deficient mice have lower BW and higher lean body mass.	Promotes both insulin secretion and insulin resistance	No changes
Cholecystokinin (CCK)	Anorexigenic ("satiety hormone")	Levels correlate directly with body weight	No major effects	No changes
Pancreatic polypeptide (PP)	Anorexigenic	Induces weight loss in animal studies. Fasting levels are lower in obese subjects and postprandial release may be exaggerated in anorexia nervosa subjects	No major effects—insulin induced hypoglycemia induces release of PP	Levels decrease or remain unchanged
Peptide YY (PYY)	Anorexigenic	Induces weight loss	Promotes glycemic control	Postprandial levels increase

Table 11.2 Possible cancer-related effects of gastrointestinal regulatory peptides

Peptide	Possible cancer-related effects	Mechanisms implicated
Ghrelin	– Increased expression in several malignancies – May promote proliferation and migration and may inhibit apoptosis	May stimulate PI3K, ERK/MAPK, Wnt-β catenin, NF-kB pathways
GIP	– Expressed in neuroendocrine tumors of GI tract and pancreatic adenocarcinoma – Stimulates growth-factor dependant pathways	May stimulate MAPK and PI3K/AKT pathways
GLP-1	– Concern with potential for incretin based therapies to cause pancreatic and thyroid cancers – Inhibits breast, prostate and colorectal cancer cell proliferation	– Stimulates pancreatic β-cell mass expansion – May stimulate calcitonin release and medullary carcinoma development in rodents
VIP	Produced at large by non-β pancreatic islet cell tumors that metastasize to the liver	VIPoma syndrome is characterized by secretory watery diarrhea, hypokalemia, and achlorhydria
CCK	– CCK receptors are expressed by tumors of neuroendocrine origin (including small cell lung cancer) – Majority of medullary thyroid carcinomas express CCK receptors	Promotes intracellular Ca++ mobilization and activation of growth promoting pathways (PLCβ, Ras, ERK, and c-jun N-terminal kinase)
Pancreatic polypeptide	Produced by pancreatic islet cell tumors (PPomas)	PPomas display a benign biological behavior
Gastrin	– Stimulates proliferation of both normal and malignant GI cells – Gastrin-secreting tumors of the duodenum and pancreas are associated with Zollinger–Ellison syndrome – Hypergastrinemia may increase risk of ECL carcinoid tumors and colorectal cancers	– Acts through Wnt-β catenin signaling, promoting increased nuclear translocation of β catenin – Attenuates PPAR-γ antiproliferative activity through EGFR and ERK1/2 activation
Polypeptide Y	– Expressed in certain carcinoid tumors – PYY agonist may decrease growth of pancreatic tumors	In ovarian carcinoids, increased expression of PYY is associated with paraneoplastic constipation
Bombesin-like peptides	– Mitogens for several human tissues – Most small cell lung cancers express bombesin/GRP	Can function as autocrine growth factors in small cell lung cancer

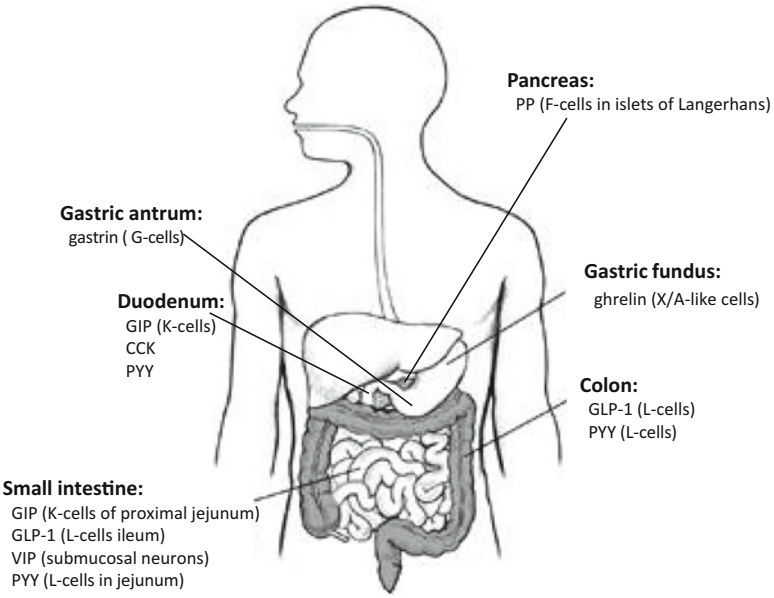

Fig. 11.1 Gastrointestinal regulatory peptides and their sites of biosynthesis by enteroendocrine cells

There are two ghrelin receptor isoforms known to date: GHSR 1a and GHSR 1b. The functional GHSR 1a isoform is expressed predominantly in the pituitary gland, with lower levels detected in other tissues, such as pancreas, myocardium, spleen, thyroid and adrenal glands [20]. GHSR 1b is the non-spliced, non-functional isoform, and has a wide distribution throughout the body [20]; its significance remains unclear.

The n-octanoyl modification of serine in position 3 is essential for the growth hormone (GH)-releasing activity of ghrelin [14]. Acylated ghrelin is involved not only in GH release and feeding behavior, but also plays an important role in the gut–insulin axis and thus energy balance [21]. While ghrelin is known to stimulate gastric acid secretion and motility in rodents [22, 23], an effect that could account for part of its hunger regulatory function, its only established role is in meal initiation.

In rodents, intracerebroventricular injections of ghrelin strongly stimulate feeding, leading to increased body weight, while peripheral administration causes reduced fat utilization and subsequent increase in adiposity [24, 25]. Such positive effects on energy balance occur independently of ghrelin's modulation of GH secretion, as it increases feeding in genetically GH-deficient mice [24].

Several hormonal signals converge at the level of ARC in the hypothalamus for control of energy balance. Neurons located at the medial ARC express orexigenic molecules such as neuropeptide Y (NPY) and agouti-related protein (AGRP), while lateral ARC neurons express anorexigenic molecules such as pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) [26]. Following intracerebroventricular administration of ghrelin, increased *Fos* transcription is noted in NPY and AGRP neurons, signaling neuronal activation of orexigenic sites [24]. Antibodies

against NPY and AGRP abolish ghrelin-induced feeding, confirming these neuronal sites as the mediators of increased feeding behavior following ghrelin treatment. Treating AGRP$^{-/-}$, NPY$^{-/-}$ double knockout mice with ghrelin or an orally active non-peptide ghrelin agonist completely abolished its orexigenic properties [27]. Moreover, ghrelin augmented NPY gene expression and blocked leptin-induced decreased feeding, suggesting that ghrelin and leptin play antagonistic roles in feeding regulation [24].

Ghrelin plasma levels increase nearly two-fold immediately prior to meals in healthy human subject and diminish to trough levels within one hour after eating [28]. The extent of this postprandial ghrelin decrease appears to be proportional to the meal's caloric content [29]. Intravenous administration of ghrelin in healthy human subjects increased energy consumption by approximately 28%, compared to saline infusion, with no effect on gastric emptying [19].

Ghrelin and Weight Disorders

Plasma ghrelin levels are inversely correlated with BMI [30]. Furthermore, plasma ghrelin levels are elevated in cachectic individuals, including those with advanced cancer and congestive heart failure [31, 32]. Plasma ghrelin levels also increase with diet-induced weight loss [33], being significantly higher in patients with anorexia nervosa compared to control subjects [34]. In anorexic subjects, an increase in BMI led to a decrease in circulating ghrelin levels by 25% [34]. In individuals who undergo RYGB surgery for the treatment of obesity, however, weight loss is not always associated with increased circulating ghrelin levels, and some series have reported that levels in persons following RYGB are actually lower than normal-weight controls [33]. This finding has not been confirmed by all studies, with some series reporting no changes in ghrelin levels following gastric bypass surgery [35]. However, although still controversial, it is possible that lower circulating ghrelin levels in individuals who undergo RYGB surgery could contribute to suppressed appetite and more effective weight loss than gastric banding [33].

One specific syndrome in which obesity is not associated with decreased ghrelin levels is Prader–Willi syndrome (PWS). In this particular group of individuals who suffer from hyperphagia, obesity and hypothalamic disorders, fasting ghrelin levels are elevated [36] compared to obese and lean controls. In addition, the typical postprandial decrease in ghrelin release is also more pronounced in PWS patients [37].

It has been postulated that ghrelin may signal the hypothalamus by crossing the blood–brain barrier and penetration into the ARC [38], by signaling the nucleus tractus solitarius in the brainstem, which communicates with the hypothalamus through activation of GHSRs on vagal afferents [39], and by production within the hypothalamus itself [40]. Patients who undergo transection of the vagus nerve due to gastric or esophageal surgery may suffer from anorexia and weight loss, which are not responsive to parenteral administration of ghrelin. This observation supports the hypothesis that the peripheral release of ghrelin influences the hypothalamic response through a vagal afferent route [41].

Ghrelin and Glucose Homeostasis

Ghrelin appears to play a role in insulin release and glucose homeostasis [42]. In healthy human subjects, intravenous ghrelin infusion leads to impaired glucose-stimulated insulin secretion [43] and precipitates glucose intolerance. Fasting plasma levels of 1040 subjects were found to be inversely correlated with fasting plasma insulin levels, the prevalence of T2DM and insulin resistance in a multivariate analysis correcting for BMI and other factors [44]. This observation appears to contradict the fact that patients who develop T2DM are generally obese and that ghrelin levels are inversely correlated to BMI. However, it has been demonstrated that differential levels of acylated and desacylated ghrelin can be measured in obese individuals with the metabolic syndrome. In fact, while total and desacylated ghrelin levels are lower than levels in nonobese subjects, obesity accompanied by the metabolic syndrome was associated with comparable acylated ghrelin and higher acylated/deacylated ratio [45]. PWS individuals, on the other hand, who suffer from hyperghrelinemia, exhibit reduced visceral adiposity and insulin resistance compared to obese controls [46].

Targeting Ghrelin for Weight Management

In order to exert its endocrine functions through activation of GHSRs, ghrelin requires acylation with an eight-carbon fatty acid at the serine-3 position [47]. Acetylation is achieved by GOAT (*G*hrelin *O*-*A*cyl*t*ransferase), member of a family of 16 hydrophobic membrane-bound enzymes that includes Porcupine, which is responsible for the acylation of Wnt proteins [48]. GOAT mRNA is largely restricted to stomach and intestines, major sites of ghrelin production. Because of ghrelin's role in the regulation of hunger and energy balance, the development of GOAT inhibitors might constitute a viable therapeutic approach to obesity [49]. GOAT can be potently inhibited by octanoylated peptides containing the first five amino acids of the ghrelin molecule [50]. The inhibitor GO-CoA-Tat has been designed to mimic and lock the complex formed among GOAT, ghrelin, and octanoyl-CoA. The inhibitor is capable of inhibiting GOAT *in vitro* in cultured cells, as well as animals. In fact, intraperitoneal administration of GO-CoA-Tat improves glucose tolerance and reduces weight gain in wild type, but not in ghrelin-deficient, mice [51]. As expected, serum levels of acyl-ghrelin were reduced, while desacyl ghrelin levels remained unchanged.

The development of GO-CoA-Tat has shed some light on the mechanistic link between ghrelin and insulin resistance. Isolated human pancreatic islet β-cells, once pretreated with GO-CoA-Tat and challenged with glucose, exhibited a significantly higher insulin response [51]. Uncoupling protein 2 (UCP2) is expressed in islet β-cells, and its induction reduces glucose-stimulated insulin secretion [52]. UCP2 mRNA levels were significantly lower in pancreatic islet β-cells of mice treated with the inhibitor [51].

Ghrelin and Cancer (See Table 11.2)

Ghrelin is expressed in a variety of cancers and cancer cell lines. Gastric adenocarcinomas, for instance, do express ghrelin, though at lower levels than normal gastric tissue [53]. Other less common malignancies, including gastrointestinal stromal tumors (GIST)[54] and gastric carcinoids [55], also express the peptide. In colorectal cancer, tissue ghrelin was found to strongly correlate with advanced stage and BMI[56]. Ghrelin receptors have been identified in breast carcinomas, with the greatest expression found in the most differentiated tumors, and with normal breast parenchyma or benign breast tumors displaying negligible expression [57]. In another study, however, ghrelin and GHSR 1a were expressed in both normal breast tissue and cancer specimens, while truncated receptor GHSR 1b was expressed exclusively in breast carcinomas [58]. Besides gastric, colorectal, and breast cancers, several other malignant histologies have been described as expressing ghrelin [59–64].

There is conflicting data regarding the potential role for ghrelin in cell proliferation [65], with studies in different cell lines demonstrating either proliferative or antiproliferative effects, possibly due to different ghrelin concentrations utilized. In the pancreatic cancer cell line LNCaP, ghrelin treatment activated extracellular signal regulated kinase (ERK) 1/2/mitogen activated kinase (MAPK) pathway leading to increased proliferation. This effect was abolished by the use of MAPK inhibitors [61].

In normal pancreatic islet β-cells, ghrelin prevents apoptosis by the activation of PI3K/AKT and ERK-1/2 pathways [66]. PI3K/ERK 1/2 activation also may mediate the antiapoptotic effects of ghrelin in other normal cell types, including adipocytes, cardiomyocytes, endothelial cells, and hypothalamic neurons [67–69]. Ghrelin also protects alveolar macrophages from lipopolysaccharide-induced apoptosis, possibly through Wnt-β-catenin pathway activation [70]. In cancer cells, data are conflicting and seem to vary depending on the cell line being investigated. Both in endometrial cancer and pheochromocytoma cell lines [60, 70] ghrelin appears to have a demonstrated antiapoptotic effect, while in colon adenocarcinoma, it induced apoptosis by inhibiting the ubiquitin–proteasome system and activating autophagy [71]. In the ovarian cancer cell line HO-8910, the antiapoptotic effects of ghrelin were reversed by activation of the mTOR/phosphatidylinositol-3-kinase (PI3K)/AKT pathway [72].

A role for ghrelin in promoting cell migration has been demonstrated in a few malignant cell lines, including pancreatic cancer [73], gliomas [64], and gastric cancer [74], through activation of PI3K/AKT and NF-kB pathways.

Because ghrelin is ultimately a mediator of hunger and food intake, its role in the treatment of cancer-induced cachexia has been pursued as a possible target for the treatment of anorexia and muscle wasting seen in certain patients with advanced cancer. Ghrelin signaling is decreased in cancer anorexia-cachexia animal models [75], even though synthesis and secretion may be upregulated [76, 77]. Despite potential benefit, the precise role of ghrelin in cancer cell proliferation, apoptosis, and invasion needs to be further elucidated before proceeding with therapeutic trials manipulating the ghrelin–hypothalamic axis in cancer patients.

Incretins: The Enteroinsular Axis

Glucose administered orally induces a far greater increment in insulin levels when compared to intravenous glucose. In 1906, it was hypothesized that small intestinal mucosal extracts contained a hormone that could regulate the endocrine pancreas, as it was demonstrated that the administration of these extracts led to decreased urinary excretion of sugar in diabetic individuals [78]. Humoral factors called incretins are responsible for mediating such effects [79]. While multiple neurotransmitters and gut hormones potentially act as incretins, two molecules have been proven to regulate nutrient-stimulated secretion of insulin physiologically: gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [80]. They are members of the secretin superfamily of peptides and share substantial amino acid identify.

Gastric Inhibitory Polypeptide (GIP)

This 42-amino acid peptide is synthesized and secreted by duodenal and proximal jejunal K-cells [81], an ideal location for sensing nutrient ingestion (see Fig. 11.1). Differential nutrient stimulation leads to GIP secretion and mRNA expression, with fat and glucose considered its main classic secretagogues [82–84]. Circulating levels of GIP typically rise immediately after a meal, in response to gastric emptying of nutrients and duodenal acidification. More recently, however, it has been demonstrated that in male healthy subjects, GIP responses are higher within 30 minutes of protein intake than after fat ingestion [85]. This may be partially mediated through the gastric acid-stimulatory properties of protein [86]. GIP levels are highly regulated by the exposure to oral nutrients, with food deprivation resulting in decreased GIP synthesis and secretion, while chronic overnutrition leads to its increased expression and secretion [87]. Patients with malabsorption have decreased post-prandial GIP levels [88].

The human receptor for GIP (GIPR) is a G-protein-coupled receptor located in many organs and tissues, including the stomach, pancreatic islet β-cells, pituitary gland, adrenal cortex, heart, adipose tissue, and brain [89, 90]. After release into the circulation, GIP is quickly hydrolyzed at the amino terminus by dipeptidyl-peptidase IV (DPP-IV) [91], which confers it a very short half-life of approximately 7 minutes [92]. This same enzyme is responsible for the degradation of several peptides, including GLP-1, as described below.

GIP in Glucose Homeostasis and Energy Balance

In addition to inhibiting gastric acid secretion [93], GIP promotes insulin release during the postprandial period [94]. Activated GIPRs located in pancreatic islet β-cells lead to an increase in intracellular cAMP through activation of adenylyl cyclase [95]. Elevated cAMP levels activate both protein kinase A (PKA) and

exchange protein activated by cAMP2 (EPAC2)/cAMP-guanine nucleotide exchange factor (GEF) II. Once active, both PKA and EPAC2 ultimately mediate intracellular Ca^{++} release and subsequent fusion of insulin containing granules with the plasma membrane [96, 97]. EPAC2 also promotes an increase in the number of insulin-containing granules near the plasma membrane [98].

Because GIP promotes insulin release following meals, one would predict that antagonizing GIP would lead to an increase in postprandial glucose levels. However, treatment of rats with a GIP-specific receptor antagonist promoted a decrease in insulin secretion by 54 % with an associated 15 % attenuation of glycemic levels 20 min after intragastric glucose administration [99]. Other studies have corroborated these findings [100, 101]. In fact, obesity and insulin resistance are associated with an exaggerated postprandial release of GIP [102], which is not reversed by caloric restriction and weight loss.

Contradictory data exists in regards to GIP levels in subjects with overt T2DM. There have been studies showing higher fasting and post-glucose stimulation GIP levels when compared to healthy controls [103] as well as minimally lower postprandial levels of intact (not degraded by DPP-IV) GIP [104]. Another study described differences in postprandial GIP levels depending on the type of oral challenge provided to diabetics: exaggerated responses were detected following mixed meals but not with glucose alone [105].

It does appear, however, that in the setting of T2DM, insulinotropic activity of GIP is greatly diminished [106], whereby the peptide will not elicit physiological insulin responses despite supraphysiological levels. Impairment in insulin release following GIP administration has been demonstrated even in first-degree relatives of patients with T2DM [107].

RYGB surgery performed in obese patients with impaired glucose tolerance has consistently been demonstrated to produce significant weight loss, euglycemia, and normal insulin sensitivity [108, 109]. While the mechanisms leading to these improvements have not been explicated, RYGB does lead to weight loss and a reduction in insulin resistance. It has been suggested that euglycemia may occur soon after RYGB because of the effects of bypassing the proximal small intestine, which alters the enteroinsular axis by preventing ingested food from stimulating the secretion of GI regulatory peptides that modulate circulating glucose and insulin levels [108]. In particular, although some investigators have reported an increase in plasma GIP levels following RYGB, most studies [110–112] have found that circulating levels of GIP, insulin, C-peptide, and glucose all decrease after RYGB, in some cases ***before*** any weight loss is evident. Interestingly, in all these studies, plasma glucagon-like peptide-1 (GLP-1) levels increased significantly after RYGB, creating ambiguities regarding its importance as a physiological mediator of the enteroinsular axis. These observations are consistent with the hypothesis that the surgical exclusion of the site of GIP synthesis and secretion following RYGB confers weight loss benefits. Interestingly, adjustable gastric banding alone is not associated with changes in incretin levels [113].

GIP may play a central role in adipocyte mass expansion. GIPR expression on adipocytes was first demonstrated in 1998 [81]. Fatty acid incorporation into adipocytes is stimulated by GIP [114], and GIPR-deficient mice were resistant to obesity

and expansion of adipose mass when exposed to high-fat diets [115]. Interestingly, *ob*:*ob*:GIP$^{-/-}$ mice exhibit improved glucose homeostasis [115]. In fact, chronic administration of GIPR antagonists in *ob*/*ob* mice leads to increased insulin sensitivity with decreased β-cell hyperplasia [100], possibly through modulation of fat accumulation in adipocytes.

All the aforementioned findings highlight the potential role of GIP-targeted therapy in the treatment of metabolic syndrome.

GIP and Cancer (See Table 11.2)

GIP stimulates growth-factor dependent pathways, such as MAPK (mitogen-activated protein kinases) and PI3K/AKT [79], both well-known cellular messengers that stimulate cell growth by enhancing proliferation, attenuating apoptosis and advancing cellular invasion. GIP overexpression has been demonstrated in different malignant histologies, particularly in neuroendocrine tumors of the gastrointestinal tract and pancreatic adenocarcinomas [116]. The possibility of using radioligands to GIPRs as diagnostic tools is currently being explored [117]. In colorectal cancer cell lines, GIP promotes activation of both PI3K and MAKP pathways, leading to increased cell proliferation in a concentration-dependent manner [118]. Taking into consideration that GIP levels can be supra-physiologic in obesity, such findings suggest a potential link between obesity and the development of certain cancers.

Glucagon-Like Peptide 1 (GLP-1):

The glucagon gene is expressed in endocrine cells of the intestinal mucosa (see Fig. 11.1), where the primary translation peptide, proglucagon, is processed to produce two highly homologous molecules, glucagon-like peptides 1 and 2 [119, 120], oxyntomodulin [121], and glicentin, which retains the glucagon sequence and is probably inactive. While glucagon-like peptide 2 (GLP-2) has been implicated primarily in the regulation of intestinal growth [122] and mucosal integrity, the 31-amino acid GLP-1, along with GIP, is heavily involved in regulating insulin secretion and other aspects of metabolism. Its insulinotropic effects seem additive to those of GIP [123].

Plasma levels of GLP-1 are low during fasting. However, within 30 min of food ingestion, L neuroendocrine cells of the distal small intestine and colon release GLP-1 in increments of two to threefold that of basal levels [105], with the majority of all GLP-1 secreted being quickly degraded by DDP-4 [124]. Further degradation takes place in the liver [125], accounting for a very short half-life of only 2 min. Different nutrients are all capable of stimulating GLP-1 release, including carbohydrates, fats, and proteins.

GLP-1 prolongs gastric emptying time by ~50% [126]. This effect seems to be mediated by the activation of gastric-projecting neurons of the dorsal motor nucleus of

the vagus nerve [127]. Gastric emptying time is also increased after vagotomy [128]. Inhibition of gastric emptying by GLP-1 seems vital for this incretin ability to influence not only satiety but also postprandial glycemia[129]. Peak plasma glucose levels correlate with the amount of gastric contents emptied five minutes after an oral glucose load. The area under the glycemia curve during the first 30 minutes also correlates with the extent of gastric emptying at 30 minutes. In contrast, plasma glucose levels at 120 min correlate inversely with gastric emptying, as well as plasma insulin levels at 30 min [130]. These findings are compatible with the theory that rapid gastric emptying leads to more dramatic plasma glucose peaks and decrements following meals, and that emptying is closely related to insulin secretion and glycemic control.

As with the incretin GIP, the receptor for GLP-1, GLP-1R, is a G-protein coupled receptor. It is expressed in many tissues in addition to the GI tract, including the islet α- and β-pancreatic cells and the central nervous system [131]. Activation of adenylyl cyclase and resulting intracellular signaling that follows the GLP-1R activation by incretins has been previously detailed in the GIP section.

GLP-1 and Glucose Homeostasis

As previously discussed, receptor stimulation by incretins incites an immediate effect by increasing intracellular Ca^{++} levels and fusion of intracytoplasmic granules containing insulin with the plasma membrane, leading to insulin secretion.

GLP-1 also promotes pancreatic islet β-cell proliferation while protecting against apoptosis, ultimately also leading to expansion of the β-cell mass [132]. GLP-1 not only promotes insulin release, but also suppresses secretion of glucagon by pancreatic islet α-cells, in order to promote normal glycemic levels after meals. Both proinsulinotropic and antiglucagon properties of GLP-1 are abolished during situations characterized by hypoglycemia [133], highlighting the functions of this molecule as a potential safe target for the treatment of T2DM. GLP-1-deficient mice exhibit hyperglycemia following oral glucose challenge, as well as diminished insulin levels, with no abnormalities in weight or feeding behavior [134].

GLP-1 and Weight Disorders

GLP-1 is a putative anorexigenic molecule. Intracerebroventricular administration of GLP-1 inhibits feeding in wild-type mice [135] but not in GLP-$1^{-/-}$ animals [134], while the intravenous infusion of GLP-1 in healthy human subjects enhances satiety and decreases spontaneous energy intake [136]. Total area under the GLP-1 response curve is approximately 80 % lower in obese compared to lean subjects [137]. Percutaneous administration of GLP-1 in healthy obese subjects is capable of inducing a 15 % reduction of mean food intake per meal and results in weight loss; these effects are associated closely with a decreased rate of gastric emptying [138]. Increased energy expenditure possibly contributes to the weight loss seen with GLP-1 therapy, as GLP-1 agonist treatment in mice stimulates brown adipose tissue thermogenesis and adipocyte browning [139].

GLP-1 Receptor Agonists and DDP-4 Inhibitors for Treatment of Type 2 Diabetes Mellitus

Both GLP-1 and GIP are quickly degraded by DDP-4 after release. This observation provided the stimulus to design GLP-1 agonists that are resistant to DDP-4, as well as DDP-4 inhibitors for the treatment of T2DM [90]. These agents are presumed to prolong the insulinotropic effects of GLP-1.

There are currently four GLP-1 receptor agonists licensed in the USA [140]. The drugs are resistant to DPP-4 and therefore have extended activity. They are all administered subcutaneously either twice daily, or daily or weekly, with the most common adverse side effect being nausea, likely related to delayed gastric emptying. They are established currently as effective therapeutic options for reducing hemoglobin A1c (HbA1c), though the agents vary in their efficacy in achieving euglycemia and promoting weight loss. Exenatide was the first GLP-1 agonist available in the USA for clinical use. When given subcutaneously once weekly in an extended release form, the medication induces greater changes in HbA1c levels, with no increased risk of hypoglycemia and similar reductions in body weight when compared to twice daily administration. 77% of patients receiving the extended release group achieved target HbA1c levels compared to 61% in the group receiving twice daily administration[141]. Liraglutide has demonstrated superiority to sulfonylurea glimepiride as a first-line agent in the treatment of T2DM, achieving lower HbA1c levels, with no hypoglycemia and no weight gain (rather weight loss) as a side effect. Liraglutide has also been demonstrated capable of inducing human pancreatic islet β-cell proliferation in vitro.

DDP-4 inhibitors are available as oral agents given once daily. They enhance levels of both GLP-1 and GIP. Hb A1c levels are significantly decreased after monotherapy with the DDP-4 inhibitor sitagliptin, which has been attributed to improved glycemic control in both fasting and postprandial states [142]. Homeostasis model assessment of β-cell function and the proinsulin to insulin ratio is improved with sitagliptin [142]. Interestingly though, the medication does not induce weight loss as seen with the GLP-1 agonists. As with the GLP-1 agonists, because of the lack of hypoglycemic effect, DDP-4 inhibitors are safe when used in combination with metformin [143] and pioglitazone [144], with perhaps long-term improvements on glycemic control due to ultimate effects in β-cell mass expansion[145].

The use of incretin-based therapy has raised concerns regarding an increased potential for the development of pancreatitis, as well as pancreatic and thyroid cancers. Because incretins possess the capacity to increase pancreatic β-cell mass, concerns regarding the possibility of promoting the development of pancreatic cancers are legitimate. However, both animal studies and clinical data have proven controversial to date. In one particular study, GLP-1 expression was detected in less than 50% of human pancreatic tumors [146]. When treated in vitro with liraglutide, human pancreatic cancer cell lines exhibited less proliferation and enhanced apoptosis [146] through both the inhibition of AKT and ERK 1/2 pathways. In another study, although no correlations were found between GLP-1 expression in human tumors and clinicopathological features, higher levels of receptors for GLP-1

(GLP-1R) were demonstrated in lymph node metastasis, as well as in sites of perineural and lymphovascular invasion. GLP-1R knockdown reduced the proliferation, migration and invasion capabilities of a pancreatic cancer cell line[147], supporting a potential role for GLP-1 in pancreatic cancer cell aggressiveness. Concerns regarding the promotion of thyroid medullary cancers stem from rodent studies in which long-term exposure to liraglutide was associated with thyroid C-cell hyperplasia and the development of thyroid C-cell tumors. These effects are presumed to be related to stimulated calcitonin release and the upregulation of calcitonin gene expression, which ultimately leads to C-cell hyperplasia, C-cell adenoma and medullary carcinoma formation [148]. However, it is possible that this effect is species-dependent as increased calcitonin levels have not been demonstrated in primates or humans undergoing long-term treatment with this medication [148, 149]. With regard to other malignant histologies, GLP-1 has been described to inhibit breast, prostate and colorectal cancer cell proliferation, while promoting proliferation and suppressing apoptosis in neuroblastoma [150–153].

The use of exenatide and sitagliptin has been reported to increase the incidence of pancreatitis by more than 6-fold when compared to other therapies [154], while rates of pancreatic cancer for both medications were found to be greater than 2.5-fold. In terms of increased chances for reported thyroid carcinomas, only an association with the use of exenatide reached statistical significance [154]. Of note, this analysis selected four other diabetes medications (not incretins) as controls and five other types of control events, in order to account for reporting bias. A recent meta-analysis that examined 55 randomized controlled trials suggests that the incidence of pancreatitis with incretin-based therapies is low and that available evidence does not support an increased risk for pancreatitis in incretin users [139]. Keeping in mind that T2DM and obesity are risk factors for the development of acute pancreatitis, and obese people are 20% more likely to develop pancreatic cancer, more information stemming from still ongoing prospective clinical trials is needed before an etiologic role can be convincingly ascertained.

Another area of intense ongoing research is the role of DDP-4 in the immune system and the possibility that it could potentially be manipulated for the treatment of Type 1 (autoimmune) diabetes mellitus (T1DM). DDP-4 is expressed on the surface of lymphocytes (CD26), and its inhibition may downregulate the pathogenic effects of Th1 cells, while promoting the activity of regulatory Th2 [155]. Further promise on a potential for treatment of T1DM relates to the fact that incretin use is capable of promoting expansion of the β islet-cell mass.

Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylate Cyclase-Activating Peptide (PACAP)

In the early 1970s, a 28-amino acid polypeptide with very broad biological functions was isolated from the small intestine and was purported to possess systemic vasodilator effects and to regulate the smooth muscle activity, as well as epithelial cell secretion, gastrointestinal tract blood flow, and glycemic levels [156]. Soon

after the peptide was linked to the watery diarrhea (originally called the Verner-Morrison) syndrome seen in patients with certain tumors of the GI tract [157–159]. Later coined as the VIPoma syndrome, the combination of severe secretory (watery) diarrhea, hypokalemia, and achlorhydria (WDHA) was found to be associated with markedly elevated plasma VIP levels and seen mostly in patients with tumors of the non-β pancreatic islets that metastasize to the liver [160], as well as ganglioneuromas and ganglioneuroblastomas [161].

The enteric secretion of VIP is stimulated largely by the vagus nerve [162]. Once released from postganglionic neurons, VIP promotes smooth muscle relaxation [163], vasodilation [164], and increased exocrine pancreatic secretion [165]. Such functions ultimately enhance nutrient absorption.

Pituitary adenylate cyclase-activating peptide (PACAP) is a 38-residue peptide initially isolated from the sheep hypothalamus. The N-terminal sequence possesses 68 % homology with VIP [166]. PACAP is not confined to the CNS, being ubiquitously encountered in peripheral neurons supplying diverse systems such as the gastrointestinal tract, adrenal glands, and blood vessels.

VIP binds to VPAC1, VPAC2, and PAC1 receptors located in a variety of tissues, from the CNS to liver, lung, intestines [167–169] and T lymphocytes [170, 171]. VPAC2 receptors are also located in smooth muscles in the cardiovascular, GI, and testis [172]. The closely related pituitary adenylate cyclase-activating peptide (PACAP) also shares binding capacity to these receptors, with VPAC1 and VPAC2 displaying similar affinity to both VIP and PACAP, but PAC1 being at least 100-fold more sensitive to PACAP binding [173]. These receptors are GPCRs, activating adenylyl cyclase and cAMP production as an intracellular messenger once activated.

Interestingly, the VPAC1 receptors are expressed in the great majority of the most commonly occurring tumors, including breast, prostate, lung, pancreas, bladder, and lymphomas [174]. The symptoms of the WDHA syndrome produced by liver metastasis of tumors of the non-β pancreatic islets, however, is thought to be mediated by the action of VIP on normal VAPC1 receptors located in the intestinal mucosa, stimulating chloride secretion and an influx of water into the bowel lumen [173]. Gastrointestinal stromal tumors (GIST) have been described as having high expression of VPAC2 receptors (as well as bombesin and cholecystokinin receptors) [175]. The roles such receptors play in malignant cell behavior and their potential use in anticancer therapeutics remain to be seen.

VIP and PACAP Role in Energy Balance and Glucose Homeostasis

Although not as well examined in the metabolic arena as some of the other peptides previously described, VIP probably does play a role in energy balance. While the incretin hormones are responsible for regulating insulin secretion in response to food ingestion, the release of insulin prior to actual nutrient absorption taking place is thought to be mediated by parasympathetic, cholinergic nerves [176]. The

cephalic phase of insulin release is mediated by olfactory and gustatory stimuli, and probably psychological anticipation of food ingestion, leading to activation of the parasympathetic system [177]. In addition to acetylcholine, VIP [178] and PACAP [179] are neurotransmitters also released by the pancreatic ganglia, and which stimulate insulin secretion by pancreatic islets [180].

Pancreatic β cells express both PAC 1 and VPAC2 receptors [173]. Mice that are deficient in VPAC 2 receptors exhibit greater lean mass, less adiposity, and increased insulin sensitivity [181]. Conversely, in vitro treatment of rat and human pancreatic islets with a VPAC2 receptor agonist led to increased insulin secretion in response to glucose [182], with similar effects observed after treatment of rodents with VPAC2 receptor agonist.

Although both VIP and PACAP are capable of stimulating insulin secretion, [183], PACAP may possess potent insulinotropic properties [184]. The intravenous administration of PACAP is capable of inducing insulin release in healthy human subjects [185], and a PACAP antagonist was shown to decrease insulin release by 18 % 15 min after a gastric glucose load [186]. Conversely, however, PACAP-deficient mice are leaner and display greater insulin sensitivity than wild-type littermates, with the lean phenotype being completely eliminated when animals are fed a high fat diet [187].

VIP levels have been reported to be abnormally increased in women with anorexia nervosa and significantly decreased on obese women [188]. In another study looking into baseline VIP levels in 26 obese subjects who entered a 3-month weight loss program, fasting VIP levels correlated with the ability to lose weight, being normal in all subjects who were able to lose more than 10% body weight [189]. However, because VIP is a neurotransmitter that is metabolized in the liver [190], the significance and value of plasma VIP measurements are questionable. VIP release after meals is not altered in individuals who undergo gastric bypass surgery [191]. VIP-deficient mice do have lower body weight and a tendency to gain lean mass as they age, with absence of regular nocturnal/diurnal feeding [192].

Therefore, based on studies in knockout animal models, both VIP and PACAP may play a role in promoting weight gain and ultimately may promote the development of insulin resistance. Whether this information will ultimately prove clinically relevant and might lead to clinical interventions for treatment of obesity and T2DM remain to be seen.

Cholecystokinin: A Satiety Hormone

In 1928, an enteric hormone capable of stimulating gallbladder contraction in dogs was isolated and demonstrated to be released in response to decreased pH in the duodenum. It was named “cholecystokinin” (CCK) based on what was regarded as its physiological role [193]. In 1943, this same peptide was reported to stimulate pancreatic enzyme secretion and was accordingly named “pancreozymin” [194]. Because of historical precedence, the name is CCK is the preferred designation.

Decades later, that hormone was also demonstrated to induce satiety in rats [6]. Its release thus appears to have a threefold purpose: to increase nutrient absorption by stimulating the secretion of biliary and pancreatic juices, enhancing gallbladder contractility and promoting satiety by delaying gastric emptying [195]. These effects appear to be mediated by the parasympathetic nerve system, as vagotomy abolishes or reduces the satiety triggered by CKK administration in rodents [196]. Lipids, amino acids and protein seem to stimulate CKK release more potently than glucose [197].

Due to post-translational modifications, of its precursor pro-CCK, there are several molecular forms with different number of amino acids but similar physiological functions (CKK-8, CKK-33 and CCK-58 among others). CCK-8 is perhaps the most studied [197] peptide of the family to date.

CCK and Energy Balance

CCK effects in satiety are mediated mostly by CCK-1 receptors [198]. Only two CCK receptors have been described to date: CCK-1 and CCK-2, formerly called CCK-AR and CCK-BR, respectively. The CCK-1R is expressed principally in the GI tract and scarcely present in the CNS, while the opposite pattern of expression is associated with the CKK-2R. Also as GPCRs, they exert their effects by activation of phospholipase C and adenylyl cyclase with the production of cAMP as an intracellular messenger [199]. CCK-1 receptors co-localize with GHRS ghrelin receptors in vagal afferent neurons, underscoring the relationship between the two peptides in appetite regulation, as CCK administration attenuates *Fos* expression in the hypothalamic ARC-induced by ghrelin [200].

Parenteral administration of CCK-8 in lean healthy human male subjects was found to induce a restriction in food intake by an average of 122 g [201]. CCK can also ameliorate hunger sensations and the desire to eat at the same time it induces satiety in both lean and obese human subjects [202]. CCK levels were found to be abnormally low in women with anorexia nervosa, while significantly elevated in obese counterparts [188]. However, the pharmacological manipulation of CCK receptors as a therapeutic tool for the treatment of obesity has been disappointing. The CCK-1 receptor antagonist loxiglumide is capable of partially restoring gastric tonic activity and reducing satiety after intraduodenal lipid infusions [203]. However, in another study in which both lean and obese women were treated with continuous intravenous infusion of loxiglumide, food intake remained unaffected [204]. These conflicting results may stem from the fact that CCK may function principally as a short-term regulator of appetite. In concordance to these findings, CKK levels remain unchanged following both RYGB surgery and vertical sleeve gastrectomy [113].

In contrast to its important physiological role as a modulator of exocrine pancreatic secretion, CCK does not appear to significantly affect endocrine pancreatic function, as CCK-1 knockout mice have no perturbations of glucose homeostasis [205].

CCK receptors are commonly expressed by tumors of neural/neuroendocrine origin [206]. CCK has been demonstrated to stimulate the growth of several malignant cell lines, including pancreatic and small cell lung cancer [207, 208]. In small cell lung cancers, there is evidence that calcium mobilization is the key event that initiates parallel activation of different proliferation promoting pathways such as phospholipase Cβ (PLCβ), Ras/ERK, and c-jun N-terminal kinase. More than 90% of medullary thyroid carcinomas do express CCK-1 receptors, and their presence has been investigated for potential diagnostic and therapeutic interventions [209].

Pancreatic Polypeptide (PP)

First isolated from chicken pancreatic extracts in 1975 [210], this 36-amino acid peptide is produced by F-cells located in the islets of Langerhans (see Fig. 11.1) [211] and is released into the circulation [212] following meals. Other gastrointestinal peptides, such as CCK and secretin, and strenuous exercise also stimulate its secretion [213, 214]. Interestingly, opposite effects are seen on PP levels depending on the manner in which glucose is administered, as the intravenous route actually induces a decrease in PP levels [215]. As with several other gastrointestinal peptides, PP release is largely controlled by the parasympathetic cholinergic nervous system, as truncal vagotomy significantly decrease the PP response after meals [216], and atropine inhibits PP responses to insulin-induced hypoglycemia, while acetylcholine stimulates them [217].

PP belongs to the pancreatic polypeptide family of endocrine peptides, which also includes peptide YY (PYY; discussed below) and neuropeptide Y (NPY) [218], all of which contain 36 amino acids [219]. While PP is expressed mostly in the pancreas and PYY in the intestines, NPY is highly expressed in the hypothalamic ARC [220]. As do other gastrointestinal peptides, this family also binds to G-protein coupled receptors that activate adenylyl cyclase in response to ligand binding [218]. Six receptor subtypes (Y1 to Y6) have been identified to date, with Y4 exhibiting the greatest affinity for PP [221]. After being released, PP inhibits pancreatic exocrine secretion, gallbladder contraction and gastric acid secretion [222].

Pancreatic Polypeptide and Energy Balance

Though clearly playing a role in energy balance, the precise functions of PP remain to be elucidated [223]. Interestingly, transgenic mice overexpressing PP in pancreatic tissues (with low expression in the brain) had reduced food intake, leading to a postnatal mortality of 50% due to impaired milk consumption. They also were leaner and exhibited delayed gastric emptying—all traits were reversible by treatment with anti-PP antibodies [211].

Fasting PP levels are lower in obese subjects [224], including patients with Prader–Willi syndrome [225]. In anorexia nervosa subjects, both an exaggerated post-prandial secretion of PP [226] and no significant changes in its levels compared to those of healthy subjects have been documented [227]. However, decreased insulin sensitivity seems to play a role in PP levels, with obese subjects who suffer from glucose intolerance displaying a more pronounced PP secretion in response to intravenous secretin compared to healthy obese counterparts [228].

Following gastric bypass surgery, basal PP levels, as well as meal-stimulated and insulin stimulated PP levels, have been reported to be significantly lower or unchanged [229–231], depending on when levels have been measured following surgery and timing with meals.

Intraperitoneal administration of PP in mice promotes weight loss through increased energy expenditure and decreased food intake, likely by modulating the expression of both orexigenic and anorexigenic peptides [232]. Leptin-deficient (*ob*/*ob*) mice chronically treated with PP displayed improved lipid profiles and less insulin resistance [232]. Currently a number of synthetic agonists are under investigation. PP 1420, for instance, is a PP analogue that is resistant to degradation and capable of sustaining a prolonged circulating half-life (approximately 2.5 h) following subcutaneous administration [233]. This peptide has been well-tolerated in Phase I studies, but its effects on weight, appetite control and caloric intake have not yet been published.

Pancreatic islet cell tumors are capable of producing PP, with up to 70 % of patients displaying abnormally high plasma PP levels following meals [234]. Pancreatic islet tumors that only produce pancreatic polypeptide (PPomas), however, are extremely rare [235]. Interestingly, these tumors do not tend to be associated with any particular clinical syndrome and have a benign biological behavior [236].

Polypeptide Y/Peptide Tyrosine Tyrosine (PYY): A Satiety Hormone

PYY was first isolated from the porcine duodenum in 1982 [237]. Also a 36-amino acid peptide, it shares receptors with PP, as previously described, as well as several physiological properties, including its capacity to inhibit the effects of CCK on pancreatic enzyme secretion [237]. PYY is co-expressed with GLP-1 in L-cells in the distal small intestine (jejunum) and in the colon (see Fig. 11.1), where it inhibits contractility and causes vasoconstriction [238]. Fat and protein seem to promote PYY release at greater levels than carbohydrates [239, 240]. In obese hyperinsulinemic women, a high-fat meal induced a greater PYY response at 30 and 60 min, while postprandial PYY levels were significantly higher at 120 min after a high-protein meal, compared to a high-fat meal [241].

PYY exists in two molecular forms, PYY (1–36) and PYY (3–36), with the latter form being the more abundantly measured in the circulation after food ingestion. It binds principally to Y2 receptors in the ARC [242] to exert its anorexigenic function. Peripheral administration in humans reduces food intake by 33 % over 24 h [242].

Higher fasting circulating PYY levels appear to predict greater anthropometric changes in obese adolescents participating in weight loss programs [243]. They are also abnormally elevated in anorexia nervosa subjects, although they were not significantly changed in obese/morbidly obese individuals [244]. As with PP levels, obese diabetic patients have higher circulating PYY levels compared to nondiabetic obese subjects [245].

Following RYGB surgery, postprandial PYY and GLP-1 levels were found to be significantly elevated, contributing to enhanced satiety, even though leptin and ghrelin levels were similar to controls [246]. Other studies have consistently documented similar changes in PYY levels after the procedure [247, 248]. Also in patients who undergo gastric sleeve surgery, similar changes in PYY levels have been documented [245] though no changes are seen after gastric banding [245]. No correlations between PYY levels and glucose homeostasis after RYGB surgery have been described [248], underscoring the lack of an insulinotropic role for this molecule.

Parenteral administration of PYY and PYY agonists reduces food intake and promotes weight loss [249] and glycemic control [250, 251], with its effects on glucose homeostasis possibly related to GLP-1 secretion [252]. Whether PYY agonists will eventually become therapeutic tools for appetite regulation and the treatment of obesity remains to be seen. PYY agonists have also been investigated for their capacity to reduce colonic motility and secretion [253] as a therapeutic option for disorders characterized by diarrhea.

PYY is expressed in certain carcinoid tumors [254]. Interestingly, in ovarian carcinoids, its high expression may be associated with the development of paraneoplastic constipation in certain patients [255, 256]. Because of its ability to suppress pancreatic exocrine secretion, its role in controlling the proliferation of pancreatic cancer cells has been studied [257]. The use of PYY agonist BIM-4300401 was reported to decrease the mass of pancreatic tumors by greater than 60% [258].

PYY may also play a role in cancer-induced cachexia, with higher circulating levels correlating with lower BMI and higher tumor burden in certain malignancies [259].

Gastrin

In 1906, J.S. Edkins reported that extracts derived from the pyloric mucous membrane of pigs' stomachs stimulated gastric secretion when injected intravenously [260]. Gastrin—as these extracts were later called—is still considered the most potent substance known to stimulate gastric acid secretion [261]. While not possessing known metabolic and energy balance regulatory properties, gastrin will be discussed in this chapter due to its remarkable role in cancer development.

After the ingestion of a meal, gastrin release into the bloodstream from antral mucosal cells (neuroendocrine G-cells) (see Fig. 11.1) stimulates the secretion of gastric acid. This polypeptide hormone acts both directly on acid-secreting parietal cells, as well as indirectly by enhancing histamine release from enterochromaffin-like cells (ECL) [262]. Both histamine and acetylcholine are also capable of

stimulating gastric acid secretion by acting on H2 and M3 receptors—respectively—present on the parietal cell basolateral membrane [261]. As intraluminal pH decreases, somatostatin is released by antral D-cells, which leads to inhibition of further gastrin secretion by G-cells and thereby prevents the development of hypergastrinemia.

Several biologically active forms of gastrin are synthesized and released into the circulation, as a result of a series of post-translational modifications. They include the α-amidated forms gastrin-17 and gastrin-34 as well as progastrin precursor peptides, such as glycine-extended gastrin (Gly-G) [263]. Only amidated gastrin forms, however, exert a physiological role in gastric acid secretion, with gastrin-17 being the predominant peptide in the gastric antrum [264].

The gastrin receptor—isolated in parietal gastric cells—is a G-protein-coupled receptor, CCK-2R [265]. It is closely related to the cholecystokinin (CCK) receptor previously described—CCK-1R—and has binding specificity for gastrin and CCK agonists and antagonists, signaling through phospholipase C.

Prolonged hypergastrinemia can be a risk factor for the development of certain gastrointestinal malignancies (see Table 11.2). Gastrin is pro-proliferative and anti-apoptotic [266] therefore being capable of stimulating not only the growth of normal gastrointestinal cells, but also colorectal, gastric, and pancreatic adenocarcinoma cell lines, among others [267].

A state of persistent hypergastrinemia is seen in the Zollinger–Ellison syndrome (ZES), characterized by gastrin-secreting tumors (gastrinomas) located most commonly in the duodenum or pancreas, which lead to the development of duodenal ulcers and other clinical manifestations. ZES can occur sporadically or as a manifestation of multiple endocrine neoplasia-1 (MEN-1) syndrome. Enterochromaffin-like (ECL) cell proliferation in the stomachs of patients with ZES was first described in the 1970s [268]. More commonly, however, elevated gastrin levels are seen in chronic proton-pump inhibitor users, and a recent meta-analysis demonstrated a higher risk for developing ECL cell hyperplasia associated with chronic PPI use [269]. However, hypergastrinemia per se does not appear to promote the evolution of ECL cell hyperplasia into neoplasia, but rather requires the presence of additional factors, such as an underlying MEN-1 syndrome [270] and gastric atrophy associated with pernicious anemia. For instance, the risk for developing ECL carcinoid tumors is at least 70-fold greater in ZES associated with MEN-1 versus sporadic ZES [271].

A seminal study in the 1990s described an association between hypergastrinemia and a 3.9 fold increased risk for colorectal cancers [272]. Transgenic mice overexpressing progastrin and Gly-extended gastrin exhibit increased colonic cell proliferation [273] while gastrin-deficient mice demonstrate the opposite [274]. Multiple mechanisms have been proposed for the permissive role of gastrin in cancer development. For instance, the Wnt-APC-β-catenin signaling is a pathway extensively described as critical for colorectal tumorigenesis [275]. Accumulation of the β-catenin oncoprotein leads to its increased translocation to the nucleus, where it promotes the transcription of multiple proliferative genes such as c-Myc and cyclin-D1. Amidated gastrin-17 not only enhances β-catenin expression but prevents its degradation in the cytoplasm, facilitating its accumulation and nuclear

translocation [276]. Gastrin has also been reported to attenuate the antiproliferative activity of peroxisome proliferator-activated receptor gamma (PPARγ). This effect appears to be mediated through ERK1/2 and EGFR activation, ultimately leading to PPARγ degradation and decreased antiproliferative activity [277].

Bombesin-Like Peptides

Bombesin was originally isolated from the skin of the frog *Bombina bombina*. In mammals, the two bombesin-like peptides identified to date are gastrin-releasing peptide (GRP) and neuromedin B (NMB). These molecules and their G protein-coupled receptors are widely distributed in mammalian peripheral tissues, including the intestine and the central nervous system. Three bombesin receptors have been cloned: gastrin-releasing peptide receptor (GRP-R/BB2-receptor), neuromedin B receptor (NMB-R/BB1-receptor), and the orphan bombesin receptor subtype-3 (BRS-3/BB3-receptor).

Both GRP and NMB can inhibit food intake when systemically administered [278, 279], although genetically engineered mice lacking either GRP-R or NMB-R demonstrate no differences in food intake or body weight compared to their wild type counterparts [280–282]. On the other hand, BRS-3 knockout mice are mildly obese, glucose intolerant, and insulin resistant [283]. BRS-3 KO mice also exhibit a reduction in oxygen consumption and mild hyperphagia. As a result of such findings, the development of BRS-3 antagonists is sparking interest as a potential therapeutic option for the treatment of obesity and type 2 diabetes mellitus [284]. Indeed, the treatment of mice with BRS-3 agonists leads to increased metabolic rate and reduced food intake and body weight [285].

Bombesin-related peptides have also been demonstrated to function as mitogens for various human tissues (see Table 11.2). The most extensively studied and established association is with small cell lung cancers—a highly aggressive form of neuroendocrine neoplasm. Bombesin/GRP is regarded as a pulmonary growth factor, and most small cell lung cancers produce bombesin/GRP, which can function as autocrine growth factors for this malignancy [286–288]. However, GRP-R, as well as NMB-R and BRS-3, has been differentially identified in a variety of other histologies, including breast and prostate cancers, bronchial and GI tract carcinoids [289], as well as renal cell carcinomas [290].

Conclusions

Beyond its role in nutrient absorption, the gastrointestinal tract is a vital endocrine organ responsible for the production of a variety of regulatory peptides that modulate energy balance. In this chapter, we describe some of the molecules mostly studied to date with regard to appetite control, glucose homeostasis, and weight

disorders. It is clear that evolutionary redundancy in the biological properties attributed to many of these peptides constitutes a potential barrier for the development of targeted therapies exploiting their roles. However, the adoption of high-throughput data collection and analysis systems may yield more precise information regarding key molecules and pathways that can be pharmacologically manipulated for the treatment of obesity and metabolic syndrome.

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