The Ghrelin Axis—Does It Have an Appetite for Cancer Progression?

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Ghrelin, the endogenous ligand for the GH secretagogue receptor (GHSR), is a peptide hormone with diverse physiological roles. Ghrelin regulates GH release, appetite and feeding, gut motility, and energy balance and also has roles in the cardiovascular, immune, and reproductive systems. Ghrelin and the GHSR are expressed in a wide range of normal and tumor tissues, and a fluorescein-labeled, truncated form of ghrelin is showing promise as a biomarker for prostate cancer. Plasma ghrelin levels are generally inversely related to body mass index and are unlikely to be useful as a biomarker for cancer, but may be useful as a marker for cancer cachexia. Some single nucleotide polymorphisms in the ghrelin and GHSR genes have shown associations with cancer risk; however, larger studies are required. Ghrelin regulates processes associated with cancer, including cell proliferation, apoptosis, cell migration, cell invasion, inflammation, and angiogenesis; however, the role of ghrelin in cancer is currently unclear. Ghrelin has predominantly antiinflammatory effects and may play a role in protecting against cancer-related inflammation. Ghrelin and its analogs show promise as treatments for cancer-related cachexia. Further studies using in vivo models are required to determine whether ghrelin has a role in cancer progression. (Endocrine Reviews 33: 849–891, 2012)

I. Introduction

Ghrelin, a 28-amino acid peptide hormone, was first discovered in 1999 as the endogenous ligand for the GH secretagogue receptor (GHSR) (1), through which it stimulates GH release. The first evidence for the ghrelin axis was provided when synthetic peptide ligands based on the structure of enkephalins were found to stimulate GH release from the anterior pituitary (2). These GH-releasing peptides (GHRP) and nonpeptidyl secretagogues, now collectively known as GH secretagogues (GHS), were found to act through the GHSR, a seven-transmembrane domain, G protein-coupled receptor (GPCR), expressed in the hypothalamus and pituitary (3, 4). Unexpectedly in 1999, the endogenous ligand for the GHSR, ghrelin, was first isolated from rat and then human stomach (1). Ghrelin has an unusual posttranslational modification because an octanoyl group is added to the third amino acid residue, which is a serine (1). Ghrelin stimulates the release of GH from the anterior pituitary through the GHSR1a, the full-length isoform of the receptor (1). This discovery demonstrated that the control of GH release was more complex than previously recognized. It rapidly emerged that ghrelin had numerous roles beyond the stimulation of GH release.
and actions that are independent of GH. Ghrelin also potently stimulates appetite (5, 6); plasma ghrelin levels are elevated before meals and reduced after feeding (7). Since the initial discovery of ghrelin, the range of physiological and pathophysiological functions attributed to this hormone has grown rapidly and includes roles in regulating metabolism and energy balance, insulin release, inflammation, and the cardiovascular system (8–12).

In 2005, Zhang et al. (13) described a second hormone, obestatin, that is also derived from the ghrelin preprohormone. This 23-amino acid, C-terminally amidated peptide was demonstrated to oppose the effects of ghrelin on appetite and feeding. The obestatin receptor was reported to be GPR39, a GPCR closely related to GHSR1a (13). This exciting finding in the ghrelin field suggested that obestatin and GPR39 could be useful targets for the development of therapeutics for the prevention of obesity. It soon emerged, however, that these results were difficult to repeat, and many subsequent studies have failed to demonstrate that obestatin opposes the effects of ghrelin on food intake. Many studies have indicated that obestatin has little or no effect on appetite (14–20). Moreover, many studies failed to confirm that GPR39 is the obestatin receptor (21–23).

In 2008, a major advance in the ghrelin field was made by two independent research groups who described the enzyme that octanoylates ghrelin, ghrelin O-acyltransferase (GOAT) (24, 25). GOAT is a member of the membrane-bound O-acyltransferase (MBOAT) family and is encoded by the MBOAT4 gene. The discovery of GOAT has led to new insights into the function and mechanism of action of the ghrelin/GHSR1a axis. Recent studies in the GOAT knockout mouse demonstrated that the ghrelin axis plays an important role in glucose balance during caloric restriction (26). A more recent study in a number of mouse models has refuted this finding however (27). GOAT is likely to regulate the balance between acylated ghrelin and its unacylated form, which is more abundant in the circulation than acylated ghrelin (28, 29). Although unacylated ghrelin (UAG) was originally thought to be an inactive form of ghrelin, because it does not activate GHSR1a, it is now clear that it has a number of functions (30–33). The finding that ghrelin has functions in cells that do not express GHSR1a and the fact that UAG also has a number of actions led to the hypothesis that there is an alternative ghrelin receptor that mediates these effects (33–36). The identity of this receptor remains unknown.

The first suggestion that the ghrelin/GHSR1a axis could be involved in cancer progression, before the discovery of ghrelin itself, was the finding that the GHSR1a is expressed not only in normal pituitary, but also in pituitary and neuroendocrine tumors (37). Soon after its discovery, ghrelin was shown to be expressed in pituitary tumors (38–40). It was suggested that ghrelin and GHSR1a could play a role in pituitary pathogenesis through an autocrine or paracrine pathway (40) by modulating pituitary hormone release (38). Ghrelin was later demonstrated to increase cell proliferation in the rat pituitary somatotroph GH3 cell line through the ERK1/2 pathway (41).

Ghrelin and its receptor are now known to be expressed by a range of peripheral tumor types and may be an autocrine/paracrine growth factor in a number of cancers. Although it is known that many tumors express members of the ghrelin axis (Table 1), the role of ghrelin in cancer has not been extensively studied. The first functional studies indicating that ghrelin may have a role in peripheral cancers demonstrated that ghrelin treatment stimulated cell proliferation in the HepG2 hepatoma cell line (42), and it was suggested that ghrelin has an autocrine or paracrine role in prostate cancer cell lines (43). Ghrelin increases proliferation in a large number of normal cell lines (33, 36, 44–66), whereas it inhibits cell proliferation in normal vascular smooth muscle and Leydig cells (67, 68) (see Section IV.A). In cancer cell lines, ghrelin increases cell proliferation in some studies (42, 43, 69–79), but decreases cell proliferation in others (34, 80, 81). Ghrelin may also regulate cell number by influencing apoptosis, and ghrelin decreases apoptosis in the majority of cell lines and cell types studied (36, 54, 57, 60, 63–67, 69, 72, 74, 82–93). The role of the ghrelin axis in cancer has not been widely studied; however, ghrelin and ghrelin analogs are promising treatments for patients with cancer cachexia (Section V). The role of the ghrelin preprohormone-derived peptide, obestatin, has not been widely explored; however, obestatin may play a role because it has been demonstrated to increase cell proliferation or inhibit apoptosis in some cell types (94–97) and decrease cell proliferation in others (98, 99).

In this review, we will summarize findings demonstrating that ghrelin, GHGR, and obestatin are widely expressed in cancer tissues and cancer cell lines and that ghrelin may have a role in a number of processes related to cancer progression, including cell proliferation, cell migration and invasion (77), apoptosis and cell survival, and angiogenesis. Ghrelin may also play a role in modulating cancer-related inflammation. Although there are conflicting studies, some single nucleotide polymorphisms (SNP) in the ghrelin and ghrelin receptor (GHSR) genes suggest that the ghrelin axis could be associated with cancer risk (100–103). Finally, we discuss the potential for the ghrelin axis to be a useful target for the treatment of cancer cachexia through a number of processes, includ-
### TABLE 1. The expression of ghrelin and GHSR, GHSR1a, and GHSR1b isoforms at the mRNA and protein levels in human tumors and cell lines

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<th>Ghrelin Protein</th>
<th>GHSR1a mRNA</th>
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<td>Follicular</td>
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<td>Papillary</td>
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<td>Medullary cell line (TT)</td>
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<td>ARO and N-PAP cell lines</td>
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(–), No expression; (–/+), negative and positive for expression within the same study; (+), low expression levels; (+) (+), moderately or highly expressed; †, expression increased compared to normal tissue levels; ‡, expression decreased compared to normal tissue levels. A number of different results have been reported in different studies.

II. The Ghrelin Axis—Gene, Peptides, and Receptors

A. The ghrelin gene, transcription products, and peptides

The ghrelin gene (GHRL) is located on the short arm of chromosome 3 (3p26) (105) and consists of four preproghrelin coding exons (exons 1–4) and a number of 5’ exons (106) (Fig. 1). The ghrelin gene is transcribed as a preproghrelin mRNA isoform, which leads to the translation of a 117-amino acid preprohormone, preproghrelin (1). The preproghrelin signal peptide is encoded by exon 1, and the 28-amino acid peptide hormone ghrelin is encoded by exon 1 and part of exon 2 (107) (Fig. 2). C-ghrelin consists of the remaining C-terminal region of preproghrelin and is encoded by part of exon 2, and exons 3 and 4 of the ghrelin gene (108). Within the C-ghrelin coding region, the peptide hormone obestatin is encoded by exon 3 (13). Although the preproghrelin-derived peptide obestatin is thought to arise through the posttranslational cleavage of the preproghrelin hormone, novel transcripts that encode obestatin and not ghrelin have also recently been described and could provide a second mechanism for its synthesis (106).

Preproghrelin contains a 23-amino acid, N-terminal signal peptide that is cleaved from the polypeptide to form...
the 94-amino acid peptide, proghrelin. Proghrelin is processed further by proteases, prohormone convertases (PC1/3 or PC2), or furin to produce the 28-amino acid ghrelin peptide (109, 110). Acylation of ghrelin is thought to occur before proteolytic cleavage from the prohormone (110). The cleavage of the 28-amino acid ghrelin molecule from proghrelin produces the 66-amino acid C-terminal peptide, C-ghrelin, and the 23-amino acid hormone obestatin is cleaved from C-ghrelin and is C-terminally amidated (13). C-ghrelin (108) and obestatin circulate in human plasma (13).

Proghrelin can be posttranslationally modified in the endoplasmic reticulum with the addition of an octanoyl group to the third residue of mature ghrelin, a serine residue, by the newly discovered enzyme GOAT (24, 25). The GOAT enzyme is a member of the MBOAT family and is encoded by the *MBOAT4* gene. GOAT is a highly hydrophobic enzyme with eight membrane-spanning domains. It recognizes the first four amino acids of ghrelin, and it uses the substrate, octanoyl coenzyme A, which it attaches to the serine 3 residue of ghrelin through an acyl bond with its side chain (25). Not all circulating ghrelin is octanoylated, however, and the majority of plasma ghrelin (approximately 80%) is a nonacylated form, desacyl ghrelin or UAG (28, 111, 112). A ghrelin deacetylation enzyme, acyl-protein thioesterase-1 (APT1), has also been described recently, and it may convert ghrelin to UAG in the plasma (113). Ghrelin deacetylation in serum has also been correlated with paraoxonase-1, butyrylcholinesterase, and platelet-activating factor acetylhydrolase (113–115), although Satou *et al.* (116) have stated that there is only conclusive evidence for APT1 as a ghrelin deacetylase in the circulation or in serum (116). High-density lipoprotein may be a ghrelin transporter in the serum, and other lipoproteins, including low-density lipoprotein, very low-density lipoprotein, and triglyceride-rich lipoproteins may also transport ghrelin (115).

There are a number of forms of ghrelin that are acylated with groups other than octanoic acid, and acylation may be manipulated through dietary modification. Decanoylated forms of ghrelin, which are modified by decanoic acid, also occur naturally (117) and have been termed D-ghrelin (118). Unlike acylated ghrelin, levels of D-ghrelin do not appear to decrease after a meal, and the kinetics of this form of ghrelin in modulating appetite may be different from octanoylated ghrelin (118). Other forms of ghrelin have also been described, including a putative alternatively spliced isoform, which is expressed in the rat stomach and would encode prepro-des-Gln (14), which lacks one glutamine residue (29). In this splice variant, an alternative splice site in exon 2 is used, and a 116-amino acid preproghrelin peptide is translated (117). This 27-amino acid form of ghrelin is believed to have similar functions to the wild-type, 28-amino acid ghrelin peptide; this form of ghrelin stimulates GH secretion in rats (29, 119), and some cardiovascular effects have been reported (120). Another octanoylated, 27-amino acid form of ghrelin, which lacks the C-terminal arginine residue, has been isolated from human stomach tissue (117).

It has recently been demonstrated that the ghrelin gene locus has a broad transcriptional repertoire (121). This includes putative noncoding RNA transcripts from *GHRLOS*, a gene on the opposite strand of ghrelin (*GHRL*) (106, 122) (Fig. 1), and ghrelin gene transcripts with heterogeneous 5′ untranslated exons, between 20 and several hundred base pairs long (121). The length of these untranslated regions is likely to affect translational efficiency (106, 107, 123–125). A range of alternative splice variants also arise from the ghrelin gene, many of which may encode novel peptides (43, 71, 106, 126, 127). Although the functions and phenotypic implications of the majority of the sense and antisense transcripts arising from the ghrelin transcripts remain to be explored, evidence is emerging that they could play important roles in disease. For example, an exon 3-deleted ghrelin transcript and its peptide are upregulated in prostate and breast cancer (70, 127), whereas the mRNA expression of a novel intron 1-retained transcript is down-regulated in particular brain regions in Alzheimer’s
patients (128) and appears to be up-regulated in invasive ductal breast cancers (71).

**B. The ghrelin and obestatin receptors**

The GHSR gene is present on chromosome 3q26.2 (129), and consists of two coding exons, separated by a 1958-bp intronic region (130). There are two known mRNA isoforms of the GHSR, GHSR1a and GHSR1b, transcribed from the GHSR gene (3). GHSR1a is known as the functional form of the receptor (3). It is a 366-amino acid, seven-transmembrane domain GPCR that mediates many of the effects of ghrelin. The GHSR1b is a truncated GPCR, and the mRNA encodes the first five-transmembrane domains, followed by intronic sequence that encodes a unique 24-amino acid C-terminal tail (3, 130). Ghrelin does not bind or activate this form of the receptor (3), and GHSR1b has been regarded as a non-functional receptor. There is evidence that GHSR1b may have some important regulatory actions, however, and may interact with the GHSR1a to alter its expression at the cell surface and attenuate constitutive GHSR1a signaling (131, 132). Both isoforms of the GHSR are widely...
expressed. GHSR1a expression was first identified in the pituitary and hypothalamus, where it is highly expressed (3). GHSR1a expression has also been demonstrated in a number of brain regions and in a wide range of peripheral tissues, including the stomach, intestine, pancreas, spleen, thyroid gland, prostate, ovaries and testes, adrenal gland, kidney, heart, lung, liver, lymphocytes, adipose tissue, and bone (3, 43, 133–138). GHSR1a mRNA may be expressed at lower levels in other tissues compared with the pituitary, however (133). GHSR1b mRNA expression has been demonstrated in numerous tissues, including the pituitary and many peripheral tissues (133).

Although acylated ghrelin binds to the GHSR1a and signals through this receptor to exert many of its endocrine effects, including the release of GH, it is now clear that there is an alternative ghrelin receptor (36, 120, 139–146). Ghrelin has a number of actions in cell types that do not express the GHSR1a, and these effects must be mediated through the hypothesized alternative receptor. In addition, UAG has a number of effects (30, 32, 33, 69, 90, 120), but it does not displace ghrelin from the GHSR1a or signal through this receptor, indicating that UAG also signals through an alternative receptor.

It was originally suggested that the receptor for obestatin was GPR39, a receptor that is closely related to GHSR and a member of the ghrelin receptor family (13). Subsequent studies, however, have failed to repeat this work. It is now clear that GPR39 is unlikely to be the obestatin receptor (23), and the identity of the obestatin receptor remains unknown. GPR39 may have roles in regulating metabolism, however, and this receptor binds zinc ions, which stimulate signaling (147–149).

**C. Functions of ghrelin, UAG, GOAT, and obestatin**

Although ghrelin was originally discovered as an endogenous GHRP, it also has a wide range of other functions. Ghrelin plays an important role in energy homeostasis, potently stimulating appetite; increasing feeding, weight gain, and adiposity; and altering energy expenditure (5, 6, 150). Ghrelin plasma levels are elevated before meals, and levels decline after eating (5, 7). Although ghrelin circulates as an endocrine hormone, ghrelin signals to the brain from the stomach via afferent neurons of the vagus nerve, and vagotomy abolishes the effects of iv-injected ghrelin to stimulate food intake and GH release (151). Ghrelin increases gut motility in preparation for feeding and stimulates gastric acid production in preparation for a meal (152–154). Ghrelin also stimulates food-seeking behavior and olfactory sensitivity, influences taste responsiveness (155, 156), and could play a role in addiction and alcoholism (157).

Ghrelin plasma levels are largely inversely correlated with body mass index (BMI), and ghrelin levels are decreased in obese subjects compared with normal-weight subjects (158), whereas ghrelin levels are elevated in patients with anorexia nervosa and cachexia (159, 160). Patients with Prader Willi syndrome are an exception, because these patients have obesity, high BMI, hyperphagia, and high circulating ghrelin levels (161, 162). Although levels of ghrelin decrease in obesity, levels increase with weight loss, and this may lead to difficulties in maintaining weight after dieting (163).

Rodent models have been useful in dissecting the role of ghrelin, GHSR, and GOAT in appetite, energy balance, and glucose balance (164). Initial studies in ghrelin (Ghrl−/−) or GHSR (Ghsr−/−) knockout mice fed a normal chow diet failed to show a distinct phenotype, and no changes in growth, food intake, or lean and fat mass were seen (165, 166). Subsequent studies have demonstrated inconsistent findings (164). Ghrelin may play a role in obesity and the response to a high-fat diet, however (167–169). Ghrelin knockout mice are resistant to high-fat diet-induced obesity, indicating that ghrelin could be a target for interventions for obesity (168). In one study, when ghrelin knockout mice were fed a high-fat diet from 6 wk of age, they remained leaner than their wild-type counterparts, and showed higher levels of energy expenditure, decreased adiposity, reduced body weight, improved glucose tolerance, and reduced plasma triglycerides and cholesterol (168). The introduction of a high-fat diet at a young age appears to be critical because this effect was not seen in animals with the same genetic background where a high-fat diet was initially fed at 8–10 wk of age (167).

Studies in the GHSR knockout mouse have demonstrated that ghrelin mediates its effects on GH release and appetite through the GHSR (170). In a study of C57BL6J mice, food intake on normal chow was not changed. GHSR-null mice fed a high-fat diet did not eat as much as wild-type mice, gained less body weight and less body fat, and stored fewer calories (171). These mice had lower levels of energy expenditure and decreased locomotor activity (171). Double knockout mice, which do not express ghrelin or GHSR, demonstrated a clear phenotype when fed a standard diet (172). These mice showed a decrease in body weight, increased motor activity, and increased energy expenditure compared with control mice (172). In these mice, food intake, lean mass, and the pattern of feeding are not altered, however (172). In another study, young adult (16 wk) double knockout C57BL/6 mice (Ghsr−/−, ghrl−/−) were not resistant to diet-induced obesity, however, and did not show a different response to wild-type littermates (173). Discrepancies in results between these studies could be a result of the different genetic backgrounds of the mice (173).
Studies into the newly discovered ghrelin octanoylation enzyme, GOAT, have led to new insights into the function of the ghrelin axis. GOAT is frequently coexpressed with ghrelin and is present in the same cell types, including cells in the gastrointestinal tract, with highest concentrations in the stomach, and also in the pancreas (24, 174). The ghrelin/GOAT system may act by informing the central nervous system and endocrine system that high-calorie food has been ingested (175). GOAT knockout mice (Mboat4−/−), which produce UAG but not octanoylated ghrelin, did not show alterations in body weight or fat mass on a normal chow diet (175). When these mice were fed a high-fat diet for at least 8 wk, however, GOAT knockout mice had a lower body weight than wild-type mice, although their fat mass was unchanged (175). Knockout mice fed a diet rich in medium-chain triglycerides demonstrated a lower body weight and lower fat mass than wild-type, despite the fact that their food intake was higher, and this probably resulted from their higher level of energy expenditure during the light phase (175). Glucose tolerance was not significantly altered in these mice (175). Transgenic mice overexpressing the human GHRL and MBOAT4 genes in the liver and fed a diet rich in medium-chain triglycerides demonstrated an increase in plasma human acylated ghrelin, body weight, and fat mass and a decrease in energy expenditure during the light and dark phases compared with the wild-type mice, but this was not accompanied by an increase in food intake (175). Interestingly, these mice lost weight when they were returned to a normal chow diet.

In a recent study, GOAT knockout mice were unable to adequately regulate blood glucose balance during severe (60%) caloric restriction and became moribund after 7 d (26). Acylated ghrelin appeared to mediate survival in these animals by regulating GH because acylated ghrelin or GH treatment rescued them from hypoglycemia (26). Recent findings in male and female GOAT (Mboat4), ghrelin or GHSR knockout mice, and ghrelin and GHSR double knockout mice, however, indicate that GOAT may not play a role in preventing hypoglycemia in chronic caloric restriction (27). Because ghrelin is the specific target for GOAT, this enzyme is likely to be a useful target for drugs inhibiting ghrelin octanoylation and preventing the effects of ghrelin on appetite stimulation. By octanoylating ghrelin, GOAT is likely to regulate the balance between acylated and unacylated ghrelin.

In addition to its effects on the gastrointestinal system, ghrelin plays a role in regulating the reproductive, cardiovascular and immune systems. It may also have roles in the regulation of anxiety, memory, and sleep and play a role in depression (176–178). Ghrelin has a number of beneficial roles in the cardiovascular system because it improves cardiac function, including contractility, reduces blood pressure without affecting heart rate, and promotes vasodilation (179–181), and ghrelin protects cardiomyocytes and endothelial cells against apoptosis (36, 182). Studies into the role of ghrelin in insulin release have been somewhat conflicting. Ghrelin has been shown to increase or decrease insulin secretion in different models, and the mechanisms involved are also currently unclear (10, 183–185). Ghrelin also has a role in modulating the immune response, reduces circulating levels of proinflammatory cytokines, and increases the expression of IL-10, an anti-inflammatory cytokine (9, 186, 187).

UAG is also likely to have an important physiological role (175). Because it does not bind and activate the ghrelin receptor, GHSR1a, it was originally thought to be an inactive form of ghrelin (1, 29). There have been conflicting reports regarding the role of UAG on appetite, with some studies indicating that UAG reduces appetite (188, 189), whereas some studies have shown that it promotes feeding (146) and others have reported that it has no effect on feeding and appetite (190). In mice, the use of an antibody that specifically hydrolyzed the octanoyl group of ghrelin to produce UAG reduced refeeding and increased metabolic rate, suggesting that UAG does not have a potent effect on appetite (191). There is evidence that UAG plays a role in energy balance, however, because transgenic mice that overexpress UAG show a decrease in fat mass, body weight, and food intake compared with control mice (188, 192). UAG secretion is elevated in response to caloric restriction, and this appears to be highly regulated, indicating an important physiological role (175, 193). UAG may play an important role in glucose balance, mediated by an alternative ghrelin receptor (175). UAG has roles in stimulating cell proliferation and improves pancreatic β-cell survival (194). It may also oppose the effects of ghrelin on the endocrine pancreas and improve insulin sensitivity through a pathway independent of the GHSR and through an alternative ghrelin receptor (32).

Obestatin, a hormone derived from the ghrelin preprohormone, was originally described as having the opposite effects to ghrelin on appetite, with ghrelin increasing appetite and feeding and obestatin opposing these effects (13). Few studies have been able to replicate these findings to confirm a role for obestatin in appetite regulation, however (14, 15, 18, 20, 146, 195–200). Unlike ghrelin, obestatin does not appear to affect the secretion of hormones from the anterior pituitary (201). Although the role of obestatin in appetite regulation remains speculative, obestatin does appear to be an endocrine hormone in its own right. The role of obestatin in obesity also remains unclear; however, it may play a role in adipocyte function (202). Obestatin has a role in a number of processes un-
related to appetite regulation; it may improve memory performance and act as an anxiolytic. It may also play a role in the regulation of metabolism during sleep and torpor and inhibit thirst (203–206).

Obestatin also plays a role in regulating the function of the endocrine pancreas. The role of obestatin in the regulation of insulin secretion is currently controversial, however, because some studies have suggested that obestatin stimulates insulin release, whereas other studies indicate that it has no role (94, 207–209). Plasma obestatin levels are inversely correlated with insulin levels, indicating that the regulation of these hormones may indeed be related (210–212). Obestatin increases insulin secretion and activates the expression of genes associated with insulin synthesis in isolated human pancreatic islet cells (94). In contrast, studies in rats and mice have indicated that obestatin inhibits insulin secretion in response to glucose or has no effect on insulin levels under basal or fasting conditions (207–209). It may have a role in diabetes mellitus, however, because exogenous obestatin treatment improved glucose balance in a streptozotocin-induced rat model of diabetes mellitus and prevented the development of diabetes in these animals (96).

### III. Expression of the Ghrelin Axis in Cancer

#### A. The expression of ghrelin and GHSR proteins and sense and antisense transcript isoforms in normal cells and tissues and in tumors

Ghrelin mRNA and the peptide are expressed in a wide range of normal tissues, including the stomach, duodenum, and to a lesser extent in the colon, hypothalamus, pituitary, endocrine pancreas, placenta, lung, cardiomyocytes, ovaries, and testes (39, 133, 213–219). Similarly, ghrelin mRNA and protein are expressed in many cancer and tumor tissues, including pituitary adenomas; cancers of the digestive tract; in lung, thyroid, breast, prostate, ovarian, testicular, endometrial, renal, and adrenocortical tumors; and in cancers of the endocrine pancreas (Table 1) (38, 39, 43, 74, 80, 81, 127, 214, 220–233). Ghrelin is secreted from a number of different cell lines, including colorectal cancer cell lines, the AGS gastric cancer cell line, the TT thyroid cancer cell line, astrocytoma cell lines, and the human erythrocytic leukemia (HEL), HL-60, THP-1, and SupT1 leukemic cell lines, and prostate cancer cell lines (73, 75, 76, 127, 234–236). Few studies have distinguished whether cancer cells produce octanoylated ghrelin, UAG, or both. The HEL, HL-60, THP-1, and SupT1 cell lines produce both acylated and unacylated ghrelin, but proportionally more acylated ghrelin (75, 76). Ghrelin production has been described in a number of neuroendocrine carcinoid tumors, including a rare tumor arising from a tailgut cyst (237), bronchial carcinoid tumors (238), gastric and intestinal endocrine tumors (239–241), and pancreatic neuroendocrine tumors (240).

Ghrelin and obestatin protein expression has been investigated in 137 patients with a range of breast cancer grades using tissue microarrays and immunohistochemistry (242). Moderate to strong cytoplasmic immunostaining for obestatin was present in 77% of cases and ghrelin immunoreactivity in 71% of cases. Ghrelin and obestatin immunoreactivity was positively correlated with the patients’ estrogen receptor status, but negatively correlated with tumor size and grade and Ki67 staining (which is an indicator of the rate of cell proliferation). This study demonstrated that patients with tumors that expressed higher levels of ghrelin also had higher survival rates, and ghrelin could, therefore, be a useful prognostic marker for breast cancer (242).

Tissue levels of ghrelin in colorectal cancer could be informative because colorectal cancer cells were demonstrated to secrete ghrelin and produce significantly more ghrelin than normal colonocytes (73). Levels of ghrelin tissue expression were highly correlated with more advanced tumors (73). Tissue ghrelin levels were proportional to the BMI of the patient, and increased BMI and obesity are important risk factors for colorectal cancer (73). The results of this study suggest that there is a mechanistic link between obesity, ghrelin, and the development of colorectal cancer (73). In contrast, other studies in colorectal cancer have suggested that ghrelin levels may be lower in patients with colorectal cancer compared with healthy controls (243). It was suggested that ghrelin could be a useful biomarker for non-small cell lung cancer (NSCLC) because in one study, ghrelin expression was detected in a majority of the 41 lung cancer tissues examined (80).

GOAT is coexpressed in the human stomach with ghrelin and is expressed in cells in the gut and the endocrine pancreas and also at lower levels in other tissues (25). GOAT mRNA expression has been demonstrated in a wide range of normal human tissues (71, 244), correlating with the widespread expression of ghrelin (244). Expression is higher in the stomach, gut, and pituitary, but GOAT is also expressed in numerous normal tissues, including the adrenal cortex, kidney, lung, spleen, fat, pancreas, and placenta (244). Studies of the level of protein expression of GOAT and measuring the activity of GOAT in different tissues are more likely to be conclusive and physiologically relevant to our understanding of the function of GOAT in different tissues. The expression of GOAT in cancer tissues is yet to be fully investigated. GOAT expression has recently been described in breast
cancer cell lines, and mean GOAT mRNA expression was elevated in 40 ductal breast tumor specimens compared with the mean value for four normal breast samples (71). It is unclear whether increases in GOAT mRNA expression also lead to an increase in GOAT protein expression or increased enzymatic activity. Although the expression of total ghrelin has been widely investigated in cancer tissues, the balance between the expression of acylated ghrelin and unacylated ghrelin has been largely unexplored.

We have also described a number of ghrelin natural antisense transcripts that are encoded by a gene (GHR-LOS) on the opposite strand of the ghrelin gene (106, 122). Although the function of this gene is currently unknown, natural antisense transcripts are now recognized to have a range of important regulatory roles in health and disease through numerous mechanisms (245). A number of ghrelin transcript isoforms and ghrelin natural antisense transcripts are expressed in cancer. A novel exon 3-deleted proghrelin isoform, which lacks exon 3, was described in normal and malignant prostate cancer tissue and cell lines (246). This ghrelin isoform would produce mature ghrelin and a novel C-terminal peptide, but not the obestatin peptide. Using antibodies raised against the novel C-terminal peptide derived from this isoform, our studies suggested that it is expressed in breast cancer tissues with high-grade carcinoma exhibiting the strongest immunoreactivity (70).

A newly described intron 1 retained variant of ghrelin (In1-ghrelin) contains intron 1 and exon 2 of ghrelin but lacks exons 3 and 4 (71). This isoform, which encodes the first 12 amino acids of ghrelin, followed by a novel C-terminal peptide, is expressed in a range of normal tissues and is highly expressed in the thymus (71). This variant lacks the coding region for obestatin. The mean expression of the In-1 ghrelin isoform was eight times higher in ductal breast cancer specimens (n = 40) than in normal controls (n = 4) (71). Expression of this variant was highly correlated with levels of GOAT mRNA expression, and these authors suggested that this form of ghrelin could be the major substrate for GOAT in breast cancer (71). It is currently unclear whether this ghrelin isoform is translated or octanoylated, however.

The functional form of the ghrelin receptor, GHSR1a, is expressed in a large number of normal tissues and tumors. GHSR1a expression was first demonstrated in the hypothalamus and pituitary (3), and expression has also been demonstrated in a wide range of peripheral tissues at the mRNA and protein levels, including the stomach, intestine, pancreas, lung, kidney, adipose tissue, thyroid, adrenal glands, prostate, ovaries, and testes (1, 43, 133, 135). GHSR1a is also expressed in a wide range of cancer tissues (Table 1), including tumors of the prostate, breast, endometrium, and ovaries, and in pituitary tumors and astrocytomas (37, 43, 71, 219, 235, 247–251). Interestingly, GHSR1a expression is reduced in some cancer tissues compared with the normal tissues from which they are derived, and GHSR1a expression appears to be down-regulated or absent in some colorectal, adrenocortical, and breast cancers, in NSCLC, leukemia, and follicular thyroid tumors (34, 81, 214, 252, 253). In astrocytoma cell lines, however, GHSR1a is up-regulated compared with normal primary human astrocytes (235). In GH-secreting pituitary adenomas, GHSR1a expression increased with tumor size and invasiveness of tumors (251).

Although the function of the GHSR1b isoform is unclear, it is expressed in a number of cancer types (Table 1) and may be overexpressed in some cancers including prostate and breast tumors, compared with normal tissues (43, 71, 247, 252). In human adrenocortical carcinoma cell lines and a number of other tumor types, GHSR1a expression is down-regulated, and GHSR1b expression is increased (73, 252). GHSR1b is overexpressed in NSCLC cell lines and in tumor samples (254). GHSR1b is thought to form a heterodimer with the neurotensin 1 receptor, a closely related GPCR in the small ghrelin receptor gene family (254). Dimerization of these two receptors leads to the formation of a novel receptor for neuromedin, and acting through this GPCR dimer, autocrine/paracrine neuromedin stimulates cell proliferation in NSCLC cell lines (254). Although this finding has not been confirmed, the GHSR1b/neuromedin 1 receptor dimer could prove a useful target for intervention in NSCLC.

Specific binding sites for ghrelin and for GHS have been described in a number of normal tissues (255) and in prostate cancer cell lines (35), and GHSR expression has previously been demonstrated in prostate cancer cell lines (43). A recent study has demonstrated that the use of a fluorescein-labeled, truncated ghrelin (amino acids 1–18) analog could be useful as a diagnostic tool for prostate cancer (256). Signal from the fluorescein-labeled ghrelin analog was amplified using a hapten amplification technique and used to examine prostate cancer, benign prostatic hyperplasia (BPH), and normal prostate tissue from 13 patients with prostate cancer (256). Ghrelin signal was significantly higher in prostate cancer tissue specimens compared with normal tissue and BPH. Although in vivo studies are required, coupled with imaging techniques, this method could be useful in discriminating benign disease and cancer in patients (256), and this is currently problematic using prostate-specific antigen screening methods. Although these authors conclude that the ghrelin probe is binding the GHSR1a in the prostate, it may also be binding the alternative ghrelin receptor. Although the role of ghre-
lin in prostate cancer proliferation is currently unclear (see Section IV.A), short-term use of this imaging probe is unlikely to cause clinical concern (256). Novel PET imaging probes based on GHSR agonists, antagonists, and inverse agonists have been developed and may also provide the basis for targeted drug delivery for obesity and other diseases (257, 258).

B. Ghrelin (GHRL) and GHSR gene single nucleotide polymorphisms (SNP) and cancer

Although results have been conflicting and inconclusive, a number of polymorphisms in the ghrelin axis have been associated with cancer risk, supporting a possible role for the ghrelin axis in cancer. Association studies involving the ghrelin and GHSR genes and height, obesity, type 2 diabetes, and cardiovascular disease have recently been extensively reviewed (259).

A number of studies have investigated the association between SNP in the ghrelin (GHRL) or GHSR genes and breast cancer risk, with one study demonstrating a link (100). In a study of 1359 breast cancer patients and 2389 matched controls, 15 common polymorphisms in the ghrelin and GHSR genes were investigated and correlated with anthropometric data as a part of the EPIC (European Prospective Investigation into Cancer and Nutrition) study (100). A SNP, rs171407-G, in the noncoding region of the ghrelin gene (GHRL) has been associated with a 20% increase in breast cancer risk [odds ratio (OR), 1.2; 95% confidence interval (CI), 1.0–1.4] and also correlates with an increase in height (100). Although cancer risk is frequently associated with an increase in BMI, surprisingly, the rs171407-G SNP is associated with a decrease in BMI (100). A SNP (rs3755777) in the 5' promoter region of the ghrelin gene was significantly associated with a 5% increase in IGF-I levels (100). These authors also described a GHSR polymorphism, rs572169 (Arg159Arg), that conferred a 20% increased risk of breast cancer and was associated with increased height, but not an increase in BMI (100). In another study, however, no association was seen between the rs572169 SNP and breast cancer (260). This SNP has also been associated with height in a genome-wide association study (GWAS) (261) and was also modestly associated with obesity in a study of a large European cohort (1275 obese subjects and 1059 controls) (262). However, this association with obesity could not be validated in a GWAS in a German cohort (262).

The EPIC study also suggested that homozygotes with a GHSR polymorphism in the noncoding region of the gene (rs2948694) have a 2-fold increased risk of developing breast cancer (100). Although the potential mechanism of action of this SNP is unknown, this polymorphism could alter GHSR gene expression. This was a very small study (n = 18), however, and these authors have recognized that the results of this study may be due to chance and studies with larger numbers of subjects are required (100).

Other studies have shown no association between breast cancer and ghrelin and GHSR polymorphisms, however. A study in a Polish cohort with 405 breast cancer patients and 460 controls, found no association between SNP in the ghrelin gene (rs26311, rs34911341, rs696217, and a SNP in the 3' untranslated region) and breast cancer risk (260). In the same study no association was seen with the GHSR synonymous SNP rs572169 (Arg159Arg) and rs495225 (Gly57Gly) (260). Fourteen different ghrelin SNP (including rs26311 and rs696217) were not associated with breast cancer risk, or with adiposity in a study of 648 patients with breast cancer and 659 controls from the Cancer Prevention Study II cohort (263). The rs572169 and the rs171407 SNP that were associated with breast cancer in the EPIC study (100) were not investigated in this study. This study was limited by a relatively small sample size, however, and the authors note that associations between the SNP studied and breast cancer may have been missed (263).

Ghrelin and GHSR SNP have been associated with colorectal cancer, although a strong association is not supported by the current data (103). In a study of four polymorphisms in the GHSR gene and seven polymorphisms in the ghrelin gene, two tagging SNP in GHRL (rs27627, rs35683) have been associated with a decreased risk of colorectal cancer. A T allele in the rs27647 SNP showed a protective effect against colorectal cancer with an OR of 0.82 (95% CI, 0.69–0.98) for heterozygotes and an OR of 0.73 (95% CI, 0.58–0.93) for homozygotes (103). The intronic SNP rs27647 (−501A>C) may have a small impact on BMI and was associated with BMI in a Caucasian (European-American) cohort (102). A SNP in the intronic region of the ghrelin gene (rs35683) was also associated with a reduced risk of colorectal cancer (103) and has also been associated with insulin levels and obesity (262). In this study, no association was seen with the GHRL gene using four tagging SNP, and there was no correlation between colorectal cancer and five other SNP in the ghrelin gene that were tested (103). Obesity and metabolic disturbance are risk factors for colorectal cancer; the ghrelin axis plays roles in regulating metabolism, and these SNP may therefore have an indirect effect on cancer risk. However, these findings in a Czech cohort (of 680 patients and 593 controls) could not be replicated by the same researchers in a German population (of 569 patients and 726 healthy controls) (103). This is unlikely to represent a population difference because the populations are genetically very similar and environmental influences are unlikely to...
play a role (103). Ghrelin could have indirect effects on cancer risk due to its influence on BMI. The German population was younger in this study, and the effects on elevated BMI may not have affected cancer development in this group (103). Further and larger independent studies are required to determine whether this association is valid; however, these authors excluded a major role for a number of ghrelin and GHSR SNP in colorectal cancer (103).

Obesity is a major risk factor for adenocarcinoma of the esophagus and of the esophagogastric junction, and it may play a role in the pathogenesis of these diseases (264). BMI has been recognized as a key environmental factor in this disease, and gastroesophageal reflux disease, also associated with obesity, is a strong risk factor (265). Using a genome-wide approach, in a study of 335 patients with esophageal adenocarcinoma, 1330 SNP were genotyped, and important SNP were identified and adjusted for covariates and false discovery rate (265). This study identified the ghrelin SNP, rs696217 (Leu72Met), as one of the most significant SNP associated with early onset of esophageal adenocarcinoma (265). Other significant SNP in other genes were associated with apoptosis pathways (265). An earlier study investigated the association between 12 SNP associated with obesity in 260 patients with esophageal adenocarcinoma, 301 patients with esophagogastric junction adenocarcinoma, 213 patients with esophageal squamous cell carcinoma, and 1352 controls (264). In this study, candidate SNP in the ghrelin gene, rs696217 and rs4684677, were investigated (264). This study also demonstrated a positive association between the rs696217 (Leu72Met) SNP and esophageal cancer, and although the association was modest and not statistically significant (264), it supports the findings of the more recent study by Wu et al. (265). Although the functional consequence of this polymorphism is unclear, the Leu72Met SNP is within the coding region for proghrelin, but not the mature ghrelin peptide itself (266). This SNP has been associated with obesity and type 2 diabetes mellitus, but results of different studies have been very inconsistent (259). Further studies into the association between SNP in the ghrelin gene and esophageal carcinoma are, therefore, warranted.

SNP in the ghrelin gene have also been associated with risk for non-Hodgkin lymphoma (101), and this disease has previously been positively associated with BMI (267). In a study of 458 patients and 812 controls, the linked ghrelin gene SNP rs1629816 (4427G>A in a noncoding region) and rs35684 (5179A>G in exon 1 of the gene) were associated with an approximate 65% decreased risk of non-Hodgkin lymphoma in homozygotes and a reduced risk for homozygotes with diffuse large cell lymphoma (101). Although this study involved a relatively small number of participants, particularly in the diffuse large cell lymphoma homozygote subgroup, and the CI were large, these results justify larger studies to validate the correlation between ghrelin gene SNP and this disease (101). Because ghrelin has antiinflammatory effects and ghrelin is chronically decreased in obesity, low ghrelin levels in obesity could increase the risk of developing non-Hodgkin lymphoma (101).

Few studies have demonstrated associations between SNP in ghrelin and/or GHSR genes and cancer risk. Associations with alleles may be due to chance, may reflect that the allele is functional, or may be due to the fact that the allele is in linkage disequilibrium with a locus that influences phenotype (259). Associations between cancer and SNP in the ghrelin and ghrelin receptor genes do not, therefore, necessarily indicate a functional role. The functional effects and biological and functional significance of a number of SNP in the ghrelin axis have not been established, and many of these are in noncoding regions. SNP in noncoding regions and synonymous SNP may influence phenotype through regulatory mechanisms.

There have been no reports of any associations between the ghrelin gene and human traits or the GHSR gene and cancer in the National Human Genome Research Institute GWAS database (268). A locus in the GHSR gene is one of hundreds of variants that has been associated with human height in a GWAS of over 100 000 individuals, the strongest association being with the synonymous SNP rs572169 (261). Studies into the relationship between SNP in the ghrelin and GHSR genes have been conflicting, however (262). GWAS have also found an association between chromosome 3q24–28 and increased risk of obesity and diabetes, and this includes the GHSR locus (3q26.31) (259). Amplifications of this region (3q26.2-q9) have also been associated with NSCLC (269), and an amplification unit at chromosome 3q25–27 has been associated with prostate cancer growth (270). The recruitment of very large cohorts for GWAS may lead to the identification of an association between ghrelin, GHSR, and disease traits (259). Many cancers, including breast and prostate cancer, are highly heterogeneous diseases, with a number of distinct molecular phenotypes (271, 272). Further stratification of patient groups to reflect their molecular subtypes may lead to more informative genome-wide studies in the future.

C. Epigenetic changes in the ghrelin axis in cancer

Epigenetic alterations and site-specific hypermethylation of promoter regions are typical features in cancer (273). Using a genome-wide approach, targeting high frequency DNA methylation changes in nine invasive ductal breast tumors, and comparing these to normal breast tis-
sue, a locus associated with the GHSR gene promoter region was shown to be highly methylated in breast cancer (274). Validation studies in 103 samples of invasive ductal carcinoma (and 104 benign or normal controls) demonstrated that the methylation status of the GHSR could distinguish between invasive ductal carcinomas and normal breast tissue with 90% sensitivity and 96% specificity (274). The authors noted that this locus was the most sensitive and specific biomarker for breast cancer reported and is an exceptionally sensitive tumor-associated DNA methylation-based biomarker (274). Methylation of promoter regions is an important mechanism that can regulate gene transcription and cell-specific mRNA expression (275). Hypermethylation of the gene led to the down-regulation of GHSR1a expression (in the small number of breast cancer samples tested); and using quantitative RT-PCR, GHSR1a expression could not be detected in the four breast cancer samples but was present in normal breast tissues (274). GHSR methylation could be a useful biomarker for breast cancer and allow the discrimination of new molecular subtypes of breast cancer (274). As discussed in Section III.A, GHSR1a expression is also reduced in a number of other cancer types compared with normal tissues, including colorectal, adrenocortical, and follicular thyroid tumors, NSCLC, and leukemia, and it would be useful to investigate the methylation status of the GHSR promoter in these cancers (34, 73, 81, 214, 252, 253).

The functional significance of GHSR1a hypermethylation and down-regulation in breast cancer is currently unknown; however, it has been hypothesized that it could play a role in tumorigenesis (274). If a causal link was proven, it is conceivable that preventing methylation of the receptor or demethylation of the receptor could lead to altered function and may affect cancer progression. DNA methyltransferase inhibitors are promising new therapeutic agents that target epigenetic alterations in cancer, and they may be used to reactivate tumor suppressor genes (276). These drugs are currently highly nonspecific, however, and have a range of adverse side effects (273). GHSR methylation could also regulate the balance between expression of GHSR1a and the GHSR1b isoform, which does not bind ghrelin. In a small immunohistochemical study, we demonstrated that GHSR1b expression was not present in normal breast glandular tissue, but was up-regulated in breast cancer specimens (70). Differential expression of GHSR1a could partly explain discrepant results in proliferation assays in breast cancer cell lines (see Section IV.A). In one study, MDA-MB231 cells were shown to express GHSR1a and proliferate in response to ghrelin treatment (70), whereas in another study MDA-MB231 cells did not express GHSR1a, and ghrelin treatment reduced cell proliferation (34). Although the expression of GHSR1a may regulate the response of cells to ghrelin (275), some of the effects of ghrelin and desacyl ghrelin are mediated through an alternative ghrelin receptor.

A recent study in rodent cell lines also suggested that the expression of GHSR1a is regulated by methylation of CpG islands in the promoter region of the GHSR gene, and these CpG island methylation sites are conserved in rodents and humans (275). In the rodent cell lines studied, hypermethylation of CpG regions in the GHSR promoter corresponded with low or absent GHSR1a expression, whereas in cell lines where the promoter region is hypomethylated, GHSR1a mRNA was expressed (275). Treatments to demethylate DNA led to an increase in GHSR1a expression in cell lines (275). Although these studies indicated that the relative expression of GHSR1a in different cell lines is regulated by GHSR promoter methylation, studies in normal tissues were inconclusive, and further studies are required (275).

D. Plasma ghrelin levels and cancer

Elevated ghrelin plasma levels have been detected in some studies in a number of cancer types (277, 278), whereas some studies have shown no correlation (73). Plasma ghrelin levels are largely inversely correlated to BMI, and because changes in circulating ghrelin levels are correlated to nutritional status, this is likely to greatly limit the use of ghrelin as a biomarker for cancer. Ghrelin levels are elevated before meals and decrease with feeding (7, 279). The significance of ghrelin plasma levels should not be interpreted without taking the nutritional status and the BMI of the subject into account; however, few studies have corrected for BMI. Ghrelin levels are frequently elevated in patients with cancer cachexia (73, 159, 280, 281), and ghrelin levels are reduced in most cases of obesity (158), with the exception of Prader-Willi syndrome, where ghrelin is greatly elevated (162). In cancer, altered ghrelin levels are more likely to be indicative of the metabolic state of the patient, rather than being directly related to the cancer itself (73), and gastric ghrelin may be secreted in response to a negative energy balance associated with cachexia (282).

The accurate measurement of plasma acylated ghrelin levels is critically dependent on the protocols used in the preparation of samples and the assays used (116). Different assays may lead to different measurements of plasma ghrelin levels (283–285). There are a number of limitations that must be considered when interpreting measurements of plasma ghrelin, including the lack of international standards, limitations of some antibody-based methods, and the rapid deacetylation and degradation of ghrelin in samples. Acylated ghrelin is quickly degraded in
the plasma, with the rapid removal of the acyl modification, and therefore, the collection and processing of blood samples is crucial to the accurate measurement of acylated ghrelin (116, 286). The half-life of the ghrelin acyl modification in the plasma is short, with a 2.9-h half-life measured for endogenous ghrelin at room temperature, a half-life of 4 h at 37 °C in ghrelin-spiked serum samples in one study, and a 45-min half-life reported in another study using plasma (114, 193, 287). Ghrelin is also degraded into C-terminal fragments by proteolysis, and a number of biologically inactive cleavage products were identified after ghrelin (amino acids 1–23) was incubated with stomach, liver, and kidney tissue homogenates (114). Although deacetylation, but not peptide degradation, was observed when ghrelin peptide was exposed to serum for several hours in this study (114), another study has indicated that ghrelin is rapidly degraded into smaller peptides in bovine plasma (116).

Because ghrelin degradation is inhibited by acidification and the acyl group is easily removed at a neutral pH, it is recommended that plasma is rapidly acidified using HCl (193, 288), although some researchers have suggested that acidification is not necessary (287). In a number of published studies, it is not clearly specified whether or not plasma samples were acidified and whether the acyl modification was largely maintained, and therefore, it is unclear in many studies whether the proportion of acylated ghrelin measured is accurate (289). The addition of 4-(2-aminoethyl)-benzene sulfonyl fluoride to the sample and preventing coagulation with EDTA and aprotinin can also prevent ghrelin deacetylation (116, 193, 287, 288).

There is evidence that both acylated and unacylated forms of ghrelin should be assayed, particularly given the fact that UAG is likely to have unique functions (193). The ratio of acylated ghrelin and UAG can be altered while the level of total ghrelin remains constant, and therefore, assaying total ghrelin alone is likely to be less informative (193, 283, 290). For example, with long-term fasting, total ghrelin levels remain the same, whereas the ratio between ghrelin and UAG is altered and acylated ghrelin levels decline (193). In some studies in cancer, acylated ghrelin levels are elevated while total ghrelin levels are unaltered (278). The measurement of total ghrelin levels has some advantages, however, because collection methods are less critical, as the acylation of ghrelin does not need to be preserved, but this method is less specific because degradation products are also likely to be detected (283). A number of studies have measured total ghrelin levels alone in cancer patients (291), whereas others have measured acylated ghrelin and total ghrelin (277).

There are no available international standards for the measurement of ghrelin, and results from different laboratories and different assays can vary widely. Different results may be obtained using different assays or assay methods (193, 283, 285, 292), with 2-fold and up to 10-fold differences in ghrelin levels from the same samples being reported between some assays (285, 288, 293). A number of factors are likely to affect the sensitivity of antibody-based ghrelin assays (283). This includes interference by plasma proteins and other molecules through nonspecific competition (283). Specific competition with ghrelin degradation products, including inactive C-terminal fragments, can be detected in single-site assays (including RIA) and could account for up to 60% of the total ghrelin measured in single-site RIA (193, 283, 294). This source of interference is minimized by the use of sandwich assays, however, where ghrelin must be recognized by antibodies to two different epitopes, leading to a more specific assay (283). Therefore, RIA have lower specificity and are less likely to detect more subtle changes in ghrelin levels than two-site assays (283). To date, most studies in cancer patients have been performed using single-site assays. In a comparative study, higher ghrelin levels were measured in a single sample tested using a single-site assay compared with a sandwich assay, and this is likely to be due to the measurement of ghrelin fragments in the single-site assays (283). Because ghrelin binds to plasma proteins, including high-density lipoproteins, the epitopes recognized may not be available to the antibody; however, this could be reversed by acidification of the sample (283). Hemolysis has also been demonstrated to affect the measurement of acylated ghrelin in a sandwich immunoassay and may also lead to variations in ghrelin measurements (295).

Several studies have investigated ghrelin plasma levels in patients with prostate cancer. In a very small study of 18 patients with prostate cancer, 12 patients with BPH, and 16 healthy controls, fasting acylated and total ghrelin levels were measured using Linco immunoassays (277). Serum levels of acylated ghrelin and the acyl ghrelin to total ghrelin ratio were significantly higher in patients with prostate cancer compared with patients with BPH or normal prostates, whereas the levels of total ghrelin were similar in all groups (277). In this study, ghrelin levels were not correlated with cachexia or reduced BMI, suggesting that this effect was not due to metabolic status (277). It is not clear whether the samples were acidified in this study. In a small study of 30 patients with prostate cancer and 50 patients with BPH, differences in total fasting ghrelin (measured using a competitive ELISA) were seen between these two patient groups, and BMI was not significantly different between the two groups (291). No correlation was observed between circulating total ghrelin or acylated ghrelin and testosterone levels in a cohort of 18 prostate...
cancer patients receiving two different hormone-suppressing treatments and a control group of 40 participants (296). Although studies linking levels of acylated ghrelin and prostate cancer have been conflicting and have investigated small cohorts, the results of these studies indicate that larger studies may be warranted.

Levels of acylated ghrelin were significantly higher in a cohort of 39 uterine leiomyoma patients than in the control group of 32 healthy patients, whereas levels of total ghrelin were not altered (278). Changes in acylated ghrelin levels were not correlated with BMI or cancer cachexia in this study (278). These assays were performed using immunoassays (278), and although assays were performed according to the manufacturer's instructions, it is unclear whether samples were acidified at the point of collection to stabilize the octanoyl modification. In a small study of 22 patients with benign ovarian tumors and 31 patients with ovarian cancers, acylated ghrelin levels were higher in patients with tumors than in the control group (n = 32), whereas levels of total ghrelin were similar in all groups (289). The ratio of acylated to total ghrelin was also elevated in these patients. These authors concluded that elevated plasma levels may indicate that ghrelin plays a role in the progression of these cancers (278), although larger studies are required.

Levels of ghrelin are elevated in medullary thyroid carcinoma tumor tissues; however, in 22 patients with medullary thyroid carcinoma, plasma ghrelin levels (measured using RIA) were comparable to those in patients with benign thyroid disease (nontoxic goiter) matched for BMI (297). These authors concluded that ghrelin was unlikely to be a useful biomarker for this disease (297).

A number of studies have measured total ghrelin levels in colorectal cancer patients (73, 243, 282), with one small study showing an inverse correlation between colorectal cancer and plasma ghrelin (243). In a study of patients with gastric and colorectal cancer, 31 patients were considered to be cachectic based on weight loss, whereas 47 patients were noncachectic, and there were 24 healthy controls (282). Fasting blood total ghrelin levels were measured using RIA (282). In this small group of patients, ghrelin levels were not correlated with nutritional status, and there was no significant increase in plasma ghrelin levels in the small groups of patients with cachexia for either cancer type (282).

It has been suggested that ghrelin may have a role in the pathogenesis of gastric cancer, and tissue levels of ghrelin may be useful as a prognostic or diagnostic marker (73). Because the stomach is the major source of ghrelin, accounting for approximately 80% of ghrelin in the circulation, treatment of gastric cancer with total or partial gastrectomy reduces circulating levels of ghrelin (298, 299). Surgery for gastric cancer may require total, distal, or partial gastrectomy, depending on the site and extent of the tumor (299). A decrease in the level of plasma ghrelin in patients with the disease could contribute to cancer cachexia, and ghrelin replacement therapy may be useful in these patients to protect against these effects (282) (see Section V). Gastric ghrelin expression is also reduced in cases of atrophic gastritis associated with chronic Helicobacter pylori infection (300), and the number of ghrelin-producing cells in the stomach decreases with the progression of chronic gastritis (301). In a study of 261 patients with gastric non-cardia adenocarcinoma, 98 patients with esophagogastric junctional adenocarcinoma, and 441 controls, total fasting ghrelin levels (measured by RIA) in the lowest quartile were correlated with an increase in cancer risk (302).

Few cases of ghrelinoma, where ghrelin is secreted at high levels in the circulation, have been described. A small number of neuroendocrine tumors, including carcinomas of the pancreas, gallbladder, and parts of the gastrointestinal tract, including the stomach and rectum, have been described where patients have exhibited high plasma ghrelin levels resulting from ghrelin hypersecretion (240, 303, 304). A transgenic mouse model of ghrelinoma has been
developed, and these mice have high levels of circulating ghrelin and exhibit hypertrophy of the stomach wall (305). Chronic hyperghrelinemia in transgenic mice stimulates the GH-IGF axis, and these mice show elevated levels of IGF-I (305). Interestingly, IGF-I is strongly correlated with an increased risk of a range of cancers, including colorectal, breast, and prostate cancer, and a number of anticancer therapies targeting the IGF axis have been developed (306). Because IGF-I is strongly correlated with cancer progression (306), this model would be useful for studying the links between the ghrelin axis and cancer development (305).

In the majority of studies, ghrelin levels are elevated in cancer cachexia; however, the mechanisms leading to elevated ghrelin levels are currently unclear (307). Elevated total plasma ghrelin levels have been reported in patients with cachexia associated with breast, colon, and lung cancer (73, 159, 280, 281). Plasma ghrelin levels are also elevated in patients with anorexia nervosa (308), and this disease appears to be a ghrelin insensitivity syndrome. Cancer cachexia has previously been described as a GH-resistant state because GH levels are elevated, whereas IGF-I levels are not (309). Similarly, anorexia associated with cancer cachexia may be a ghrelin insensitivity syndrome (310). Elevated ghrelin plasma levels could be the result of a compensatory mechanism in cancer, representing an attempt to improve the body’s energy balance and to stimulate food intake (159). It has been suggested that ghrelin secretion is increased in response to inflammation during cachexia because ghrelin has antiinflammatory properties (281, 311). The symptoms of cancer cachexia are associated with increased plasma levels of proinflammatory cytokines, including TNFα, IL-1β, IL-6, and interferon γ (307, 312). Ghrelin has been demonstrated to reduce levels of TNFα, IL-1β, IL-6, and IL-8, and the proinflammatory transcription factor, nuclear factor κB (NFκB), and to increase the production of the antiinflammatory cytokine IL-10 (311) (see Section IV.D). There have been some conflicting studies, however, with some studies demonstrating unchanged or lower levels of ghrelin in patients with cancer cachexia compared with controls (282, 313, 314).

In NSCLC patients, ghrelin levels were elevated, and patients with weight loss had significantly higher levels of ghrelin (281). In this study of 76 NSCLC patients without prior weight loss, 25 patients with prior weight loss, and 60 controls, total serum ghrelin levels were measured using a sandwich immunoassay, and comparisons were made after adjustment for age, gender, and BMI (281). Ghrelin serum levels were elevated in patients with and without weight loss compared with healthy volunteers. In this study, ghrelin levels were not correlated with disease progression or with overall survival, however, indicating that ghrelin may not be a useful prognostic marker for NSCLC (281). Up to 60% of lung cancer patients present with weight loss as a symptom on diagnosis. Ghrelin could be a useful marker for cachexia in this disease, allowing early detection and treatment of this syndrome (281) (see Section V).

Similarly, in a study of gastric cancer patients with more than 10% weight loss due to cancer cachexia, mean ghrelin levels were elevated in patients compared with patients without cachexia and normal controls (315). In lung and gastric cancer patients with cachexia, plasma ghrelin levels were inversely correlated with a decrease in BMI (310, 315). In a study of patients with cancer, ghrelin levels were compared between a group of 21 patients with cancer-related cachexia, 24 cancer patients without cachexia, and 23 normal controls (310). Fasting blood samples were acidified and treated with phenylmethanesulfonyl fluoride for acylated ghrelin determination using Linco RIA (310). Acylated ghrelin levels and the acyl ghrelin to total ghrelin ratio were significantly higher in subjects with cancer cachexia compared with patients with cancer, or unaffected controls, and total and acylated ghrelin levels were inversely proportional to BMI (310). In another study of patients with gastrointestinal cancer, plasma levels of total ghrelin and acylated ghrelin were elevated; however, there was no strong inverse correlation between ghrelin and BMI in this study (307).

Although a number of studies have demonstrated elevated levels of ghrelin in some cancers, results are currently conflicting and many studies are based on small cohorts. Further optimization and standardization of ghrelin measurement techniques and the introduction of more robust assay methods is likely to lead to more consistent results that will facilitate the correlation between disease and plasma ghrelin levels. Nevertheless, plasma ghrelin levels are more likely to correlate with metabolic state and may, therefore, not be useful as a plasma marker for most cancers but could be useful for the early detection of cancer cachexia.

IV. The Role of Ghrelin in Processes Related to Cancer Progression

A. Ghrelin and cell proliferation and apoptosis

An increase in cell proliferation and the evasion of apoptosis are hallmarks of cancer (316, 317), and ghrelin may play a role in regulating cell number in normal and cancer cells by affecting cell survival, apoptosis, and cell proliferation (Fig. 3). Ghrelin stimulates cell proliferation in the majority of normal cell lines and cell types tested.
Figure 3. Ghrelin is involved in a number of processes associated with cancer progression, including cell proliferation, cell migration and invasion, and the inhibition of apoptosis, and ghrelin could play a role in angiogenesis and cancer-related inflammation.

Figure 3. Ghrelin is involved in a number of processes associated with cancer progression, including cell proliferation, cell migration and invasion, and the inhibition of apoptosis, and ghrelin could play a role in angiogenesis and cancer-related inflammation.

Table 2, including human oral keratinocytes (44), fetal skin cells (45, 46), the rat somatotroph GH3 cell line (41), a range of neuronal cell types (47–51), human and rat osteoblasts (33, 52, 53), adipocytes (54, 55), and human aortic endothelial cells (56). Ghrelin both stimulates cell proliferation and protects cells against apoptosis in normal-derived cells and cell lines including mouse MC3T3-E1 osteoblastic cells (57), pancreatic β-cells and pancreatic islet cells (58, 59), 3T3-L1 preadipocytes (54), H9c2 cardiomyocyte cells (36, 60), ovarian follicular cells (61, 62), and adrenocortical cells (63, 64). Ghrelin stimulates cell proliferation and healing in gastric and duodenal ulcers induced by acetic acid in rats (65) and increases cell proliferation and decreases apoptosis in hypotrophic gut mucosa (66). Conversely, ghrelin has been shown to inhibit proliferation in human aortic smooth muscle cells (318) and immature testicular Leydig cells in vivo (68).

Ghrelin stimulates cell proliferation by signaling through the MAPK extracellular signal-related kinase 1/2 (ERK1/2) pathway in a number of normal cell lines, including the FRTL-5 rat thyrocyte cell line (319), the normal rat somatotroph GH3 cell line (41), mouse osteoblastic cells and human osteoblasts (33, 57), and human adrenocortical cells (64). In the GH3 cell line, treatment with the nitric oxide (NO) donor, S-nitroso-N-acetylpenicillamine, reduced both basal and ghrelin-induced cell proliferation and reduced ERK1/2 activation in response to ghrelin treatment (320). In contrast, in the TE85 human osteoblastic cell line, ghrelin stimulates cell proliferation through the NO pathway, and the NO inhibitor Nω-nitro-l-arginine methyl ester inhibits the proliferative response to ghrelin, whereas treatment with the NO donor S-nitroso-N-acetylpenicillamine stimulated cell proliferation (52).

Ghrelin stimulates cell proliferation and protects against apoptosis in the normal gut, including the small intestine in a hypotrophic gut mucosa rat model (66), and ghrelin has protective effects in the gut against a wide range of insults (321). Although fasting in rats stimulates mucosal cell apoptosis, cerebroventricular infusion or injection of ghrelin reduced apoptosis in the intestinal mucosa, demonstrated by a decrease in terminal deoxynucleotidyl transferase dUTP staining, DNA fragmentation assays, villous height and caspase 3 expression (88). Bromodeoxyuridine staining indicated that ghrelin treatment also increased cell proliferation in the intestine (88).

UAG has similar effects to ghrelin, increasing cell proliferation in a number of normal cell lines, including the SW-13 and NCI-H295R adrenocortical cell lines, in the GH3 somatotroph cell line, in human osteoblasts, in the rat INS-IE and hamster HIT-T15 β pancreatic cell lines, and in human pancreatic islet cells (10, 33, 41, 59, 69, 194). UAG also increases the mass of the pancreatic islets in the streptozotocin-treated diabetic rat model, preventing the development of diabetes (96).

Ghrelin may act as an autocrine/paracrine growth factor in some cancers (43, 246). Ghrelin stimulates cell proliferation in most of the normal cell types tested, and therefore, it is not surprising that ghrelin also increases proliferation in a number of cancer cell lines. Ghrelin has been demonstrated to decrease cell proliferation in some cancer cell types, and some conflicting findings have been reported in different studies using the same cell lines (Table 2). Ghrelin may play a role in promoting pancreatic cancer because exogenous ghrelin treatment stimulates cell proliferation in the PANC1, MIAPaCa2, BxPC3, and Capan2 pancreatic cancer cell lines (77). This response was inhibited by the GHSR1a inverse agonist, D-[Lys-3]-GHRP-6, demonstrating that this effect is likely to be mediated through the GHSR1a (77). In endometrial cancer cell lines, acylated ghrelin treatment increases cell proliferation and also protects against apoptosis induced by doxo-
### TABLE 2. The effects of ghrelin on cell proliferation, apoptosis, and cell migration and invasion in normal cell lines and cell types and in tumors and cancer and tumor-derived cell lines

<table>
<thead>
<tr>
<th>Cell type</th>
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<th>Apoptosis</th>
<th>Migration and/or invasion</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>Adipocytes</td>
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<td></td>
</tr>
<tr>
<td>3T3 preadipocytes</td>
<td>↑ (54, 55, 397)</td>
<td>↓ (54)</td>
<td></td>
</tr>
<tr>
<td>Adrenocortical cells</td>
<td>↑ (63, 64)</td>
<td>↓ (64)</td>
<td></td>
</tr>
<tr>
<td>Breast cell lines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF10A normal-derived</td>
<td>N (70)</td>
<td></td>
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</tr>
<tr>
<td>Cardiomyocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9c2 cardiomyocytes</td>
<td>↑ (60)</td>
<td>↓ (36, 62, 341)</td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic endothelial cells</td>
<td>↑ (56)</td>
<td>↓ (36, 182)</td>
<td></td>
</tr>
<tr>
<td>HUVEC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>HMVEC</td>
<td>↑ ↓ (339)</td>
<td>↓ (87)</td>
<td>↑ (339)</td>
</tr>
<tr>
<td>Pancreatic islet endothelial cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat CMEC</td>
<td>↑ (365)</td>
<td></td>
<td>↑ (365)</td>
</tr>
<tr>
<td>Gut cells</td>
<td></td>
<td></td>
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<tr>
<td>Neuronal cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>↑ (61, 62)</td>
<td>↓ (61, 92)</td>
<td></td>
</tr>
<tr>
<td>Ovarian follicular cells</td>
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<tr>
<td>Pancreatic cells</td>
<td></td>
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<tr>
<td>β-cells and islet cells</td>
<td>↑ (59)</td>
<td>↓ (58, 87, 399)</td>
<td></td>
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<tr>
<td>INS-IE and HIT-T5 cells</td>
<td>↑ (59, 194)</td>
<td>↓ (59, 194)</td>
<td></td>
</tr>
<tr>
<td>Pituitary cells</td>
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<td></td>
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</tr>
<tr>
<td>ACTH pituicytes</td>
<td>↑ (400)</td>
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</tr>
<tr>
<td>Rat diabetic lactotrophs</td>
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</tr>
<tr>
<td>Skin cells</td>
<td>↑ (44–46)</td>
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</tr>
<tr>
<td>Keratinocytes and skin</td>
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<tr>
<td>Testes</td>
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</tr>
<tr>
<td>Immature Leydig cells</td>
<td>↓ (68)</td>
<td></td>
<td></td>
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<tr>
<td>Vascular smooth muscle</td>
<td>↓ (67, 318)</td>
<td>↓ (67)</td>
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</tr>
<tr>
<td><strong>Tumor-derived cell lines</strong></td>
<td></td>
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<tr>
<td>Astrocytoma</td>
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<tr>
<td>CCF-STT1G1, U-118, U-87, SW1088 cell lines</td>
<td>↑ (235)</td>
<td></td>
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<tr>
<td>Adrenocortical tumors</td>
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<tr>
<td>NCI-H295R cell line</td>
<td>↓ (252), ↑ (69)</td>
<td>↓ (69), ↑ (328)</td>
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<tr>
<td>SW13 cell line</td>
<td>↓ (252), ↑ (69)</td>
<td>↓ (69), ↑ (328)</td>
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<tr>
<td>Aldosterone</td>
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</tr>
<tr>
<td>Breast cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF7</td>
<td>↓ (34), N (70)</td>
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<tr>
<td>MDA-MB231</td>
<td>↑ (70), ↓ (34), ↑ In-1 (71)</td>
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<tr>
<td>MDA-MB435</td>
<td>↑ (70)</td>
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<tr>
<td>Choriocarcinoma</td>
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<tr>
<td>JEG-3 choriocarcinoma cell line</td>
<td>↑ (72)</td>
<td>↓ (72)</td>
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<tr>
<td>Colorectal cancer</td>
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<tr>
<td>SW-48, RKO cell lines</td>
<td>↑ (73)</td>
<td></td>
<td>↑ (73)</td>
</tr>
<tr>
<td>HT-29 cell line</td>
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<tr>
<td>Endometrial cancer</td>
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<tr>
<td>Ishikawa, HEC1B, KLE cell lines</td>
<td>↑ (74)</td>
<td>↓ (74)</td>
<td></td>
</tr>
<tr>
<td>Esophageal cancer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OE-19/esophageal adenocarcinoma cells</td>
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<tr>
<td>Leukemia</td>
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<tr>
<td>Human erythroleukemic cell line (HEL)</td>
<td>↑ (75)</td>
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<td></td>
</tr>
<tr>
<td>HL-60 and THP-1 cells</td>
<td>↑ (76)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver tumor</td>
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<td></td>
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<tr>
<td>Hepatoma cell-line HepG2</td>
<td>↑ (42)</td>
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<td></td>
</tr>
<tr>
<td>Lung tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H345 small cell lung cancer cell line</td>
<td>↓ (80)</td>
<td></td>
<td>↑ (80)</td>
</tr>
<tr>
<td>Calu-1 lung epithelial carcinoma cell line</td>
<td>N (253)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreatic cancer</td>
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</tr>
<tr>
<td>Adenocarcinoma cell lines PANC1, MIA PaCa2, BxPC3, Capan2</td>
<td>↑ (77)</td>
<td></td>
<td>↑ (77)</td>
</tr>
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</table>

(Continued)
rubricin (74). Ghrelin also stimulates cell proliferation and protects against apoptosis in the JEG-3 choriocarcinoma cell line (72).

Ghrelin may play a role in gastric and colorectal cancer (73). Although normal human colonocytes produce low levels of ghrelin, expression is higher in the SW-48 and RKO malignant human colon cell lines (73). Ghrelin may be an autocrine/paracrine growth factor in these cells because treatment with the GHSR1a inverse agonist D-[Lys-3]-GHRP-6 or treatment with a ghrelin-neutralizing antibody greatly reduced proliferation in these cell lines (73). In the human HT-29 colon cancer cell line, ghrelin treatment had an antiapoptotic effect, protecting cells against the toxic effects of 5-fluorouracil, a cytotoxic drug used in the treatment of colorectal cancer (83). Ghrelin reduced the rate of apoptosis in these cells through altering the ratio of Bcl-2 and Bax proteins (83).

It has recently been demonstrated that the transcription factor KLF4 (Kruppel-like factor 4), interacts with response regions in the promoter region of the ghrelin gene (322). KLF4 has roles in stimulating genes associated with gastric epithelial cell proliferation and differentiation and is involved in p53-mediated cell cycle arrest in response to DNA damage (322). KLF4 expression in the AGS gastric cancer cell line stimulates ghrelin expression (322). Ghrelin secretion in the AGS gastric cancer cell line is influenced by lysophosphatidic acid, an extracellular lipid-signaling molecule secreted by adipocytes, which strongly decreases ghrelin secretion (236).

Although ghrelin has been demonstrated to increase cell proliferation in a large number of normal cell lines and some cancer cell lines, ghrelin inhibits cell proliferation in some cell lines. In the H345 small cell lung cancer cell line, ghrelin and UAG inhibit cell proliferation, and ghrelin has proapoptotic effects leading to a reduction in cell number (80). In the CALU-1 lung epidermoid carcinoma cell line, ghrelin treatment had no effect on cell proliferation over 24 h, but the synthetic GHS, hexarelin and EP0–80317, inhibited cell proliferation and also inhibited IGF-II-stimulated proliferation (253). Ghrelin also appears to decrease cell proliferation in the N-PAP papillary thyroid cancer cell line (81), whereas in the ARO anaplastic thyroid carcinoma cell line, ghrelin was shown to inhibit cell proliferation in one study (81), but it had no effect alone or in combination with TSH treatment in another study (319). In contrast, ghrelin treatment stimulates cell proliferation in the rat FRTL-5 thyrocyte cell line (81).

The role of ghrelin in cell proliferation in breast and prostate cancer cell lines remains unclear due to conflicting studies. In the MCF7 and MDA-MB-231 breast cancer cell lines, ghrelin was shown to decrease cell proliferation in one study, although ghrelin stimulated cell proliferation in the MDA-MB-231 cell line in different studies (34, 70, 71). Ghrelin had no significant effect on cell proliferation in the MCF7 cell line or the MCF10A normal breast-derived cell line (34, 70). A novel ghrelin isoform, In1-ghrelin, which retains intron 1, is expressed in a range of tissues, appears to be overexpressed in breast cancer, and may also play a role in cell proliferation (71). MDA-MB231 cells overexpressing the In1-ghrelin variant proliferated at a higher rate than control cells expressing vector alone (71). Interestingly, mean expression of this isoform in breast cancer samples was significantly correlated with the levels of expression of cyclin-D3, a marker of tumor cell proliferation (71). Wild-type ghrelin treatment stimulates cell proliferation in the MDA-MB-435 cell line, although there is some suggestion that this breast cancer cell line may be derived from a malignant melanoma (70). Transcriptomic studies in canine mammary cancer cell lines also support the fact that ghrelin may play a role in proliferation in breast cancer. The expression of ghrelin and GHSR1a was related to cancer cell lines with a high rate of proliferation (323). GHSR1a mRNA expression has been demonstrated in the MDA-MB-231, MCF7, MDA-MB-435, and T47D breast cancer cell lines using RT-PCR (70) but was not
detected in the MDA-MB-231 and MCF7 breast cancer cell lines in another study (34). These contrasting observations could indicate that the cell lines used in this study could be different variants, and this may explain some of the different functional results observed. Discrepancies between studies could also result from the RT-PCR assays themselves or a number of variations in the methods applied, including the types of proliferation assays used, the concentrations and purity of ghrelin applied, and the confluence of the cells at the beginning of the experiment. Ghrelin is unstable in cell culture and is likely to have a short half-life because the octanoyl group is rapidly removed and the ghrelin peptide is proteolytically cleaved (114, 288). Studies into the effects of ghrelin are, therefore, likely to require the frequent, continuous, or pulsatile application of ghrelin peptide.

Conflicting results have been observed in the study of prostate cancer cell lines. In one study in the PC3 prostate cancer cell line, cells (grown in the presence of 10% fetal calf serum) were treated with ghrelin over 72 h, which stimulated cell proliferation with 10 nM treatments (43, 127). A less significant proliferative response was seen with 1 μM ghrelin treatment. Ghrelin was replenished every 24 h in this assay, and cell number was estimated using a colorimetric, metabolic assay (127). These cells were shown to express GHSR1a. A similar response was seen in the PC3 cell line in another study, where lower concentrations of ghrelin stimulated cell proliferation, whereas higher concentrations (1 μM) inhibited cell proliferation (35). This study failed to demonstrate GHSR1a mRNA expression in this cell line, although binding sites for ghrelin were reported, indicating that ghrelin could act through an alternative ghrelin receptor in this cell line (35). Differences in expression of GHSR1a and the ratio between the GHSR1a and the alternative ghrelin receptor, even within the same cell line, could contribute to these different results. In contrast, a more recent study demonstrated that ghrelin decreased the rate of PC3 prostate cancer cell proliferation (79). In this study, PC3 cells were treated with serum withdrawal, were routinely grown in low serum media (3%), and may, therefore, have been phenotypically different (79). It is unclear whether ghrelin was replenished during the 72-h treatment period. These authors measured proliferation using tritiated thymidine incorporation in the last 6 h of a 72-h ghrelin treatment, and therefore, only cells dividing in the last 6 h of the assay would have incorporated tritiated thymidine into newly synthesized DNA and have been measured (79). The confluence of the cells at this time may have varied between the treatments, and more confluent cells may have stopped dividing in this time. Because ghrelin is rapidly inactivated in serum (114, 288), low levels of acylated ghrelin, UAG, and ghrelin breakdown products may have been present in the media after 66-h incubation. Ghrelin fragments are also known to inhibit the activity of the ghrelin acylation enzyme, GOAT (324). Indeed, the purity of ghrelin used in different assays and the presence of shorter forms of ghrelin produced during the synthesis process, or as a result of storage, could lead to discrepant results. Short-term assays using tritiated thymidine may be more informative.

Ghrelin was also shown to have a proapoptotic effect in PC3 cells (using terminal deoxynucleotidyl transferase dUTP assays), signaling through T-type calcium channels (79). These authors demonstrated expression of GHSR1a and GHSR1b mRNA and GHSR1a protein expression using immunohistochemistry (79). Although cell proliferation in response to ghrelin treatment has also been demonstrated in the LNCaP prostate cancer cell line (43), another study demonstrated that ghrelin treatment had no effect in this cell line (35). In the DU145 prostate cancer cell line, treatment with ghrelin alone had no effect on cell proliferation, but ghrelin and UAG inhibited cell proliferation in response to IGF-I (35).

Ghrelin (or GHS) may stimulate or inhibit cell proliferation in cell lines that do not express the GHSR1a, suggesting that these effects must be mediated through the hypothesized alternative ghrelin receptor, the identity of which remains unknown (253). For example, although the HepG2 cell line does not express the GHSR1a (139), ghrelin stimulates cell proliferation and signals through the insulin pathway (42). In the HEL, HL-60, and THP-1 human leukemic cell lines, ghrelin appears to play a role in stimulating cell proliferation, although these cell lines do not express GHSR1a mRNA (75, 76). This may be an autocrine effect because these cell lines produce and secrete ghrelin and proliferation is inhibited by treatment with two different antighrelin antibodies. This effect appears to be mediated by the alternative ghrelin receptor (75, 76). The addition of exogenous acylated or unacylated ghrelin did not affect cell proliferation in the HL-60 and THP-1 cell lines, however (76). In addition, UAG, which does not activate the GHSR1a, also stimulates cell proliferation in some cell lines, and these effects must be mediated by an alternative ghrelin receptor (33, 69).

There have been few in vivo studies into the role of the ghrelin axis in cancer, although the ghrelin axis has been targeted as a treatment for cachexia (see Section V), and this has been investigated using mouse and rat models. Two studies investigating the effects of ghrelin treatment on cachexia in mouse and rat tumor-bearing models demonstrated that short-term treatment did not lead to an increase in tumor size (325, 326). A transgenic model of ghrelinoma, using the ghrelin promoter and an SV40 T antigen, has been developed to investigate the chronic ef-
flects of ghrelin overexpression (305). These mice demonstrated hypertrophy of the stomach mucosa from 9 wk of age and the development of ghrelin-producing tumors (305). These animals also had elevated levels of IGF-I, independent of nutritional status (305), and chronically elevated IGF-I levels could promote the development of cancer. Other studies have not reported an increase in cancer risk in transgenic mice overexpressing ghrelin or desacyl ghrelin, although this may not have been investigated (192, 327).

Ghrelin may protect against apoptosis in a number of cancer cell types, while increasing apoptosis in others. In the SW-13 adrenocortical carcinoma cell line, UAG treatment reduced basal apoptosis and had a more protective effect against basal apoptosis than acylated ghrelin (69). Ghrelin may inhibit cancer progression in adrenocortical cell lines because it stimulated a marked increase in the basal apoptotic rate in the SW-13 and NCI-H295 adrenocortical carcinoma cell lines and in an aldosteroma cell line (328). In contrast, however, this effect was not seen in another study in the SW-13 adrenocortical carcinoma cell line because ghrelin reduced basal apoptosis and caspase 3/7 activity (69). Ghrelin protected against apoptosis, which was induced using sodium nitroprusside, in a rat PC-12 pheochromocytoma cell line (from an adrenomedullary tumor) (84). This was mediated by the inhibition of the proapoptotic MAPK apoptosis signal regulating kinase 1 pathway and through the induction of the heat shock protein, Hsp70 (84). In contrast, ghrelin did not affect apoptosis in Barrett’s esophageal adenocarcinoma cells treated with a number of apoptosis-inducing agents (329), and no effect was seen in prostate cancer cell lines treated with actinomycin in another study (127). Ghrelin may protect against apoptosis in a range of normal cell types in response to a wide range of insults and has a protective role in the gastrointestinal tract, the cardiovascular system, and the nervous system (89, 330–334) (Table 2). Ghrelin inhibits apoptosis by signaling through the ERK1/2 pathway in 3T3-L1 preadipocytes and in rat hypothalamic and cortical neurons (54, 89, 90).

HEK293 human embryonic kidney cells overexpressing GHSR1α were protected against apoptosis stimulated by cadmium, and this effect was not seen in cells lacking GHSR1α expression (335). It was not clear whether this is mediated through the constitutive action of the GHSR1α or through the action of autocrine ghrelin, however (335). The GHSR1α has a high level of constitutive activity and signals through the protein kinase C/phospholipase C pathway, and therefore, stimulation by the agonist ghrelin is not required for activity (336).

Although there have been few studies into the effect of ghrelin on apoptosis in cancer cell lines, most studies have reported that ghrelin protects against apoptosis (36, 54, 57, 60, 63–67, 69, 72, 74, 82–93), although in some cell types ghrelin may be proapoptotic (79, 80, 328) and ghrelin increases basal apoptosis in some cell lines (328). Ghrelin may promote cancer progression in some cell types by stimulating cell proliferation and inhibiting apoptosis and may inhibit cancer progression in others. Different responses in different cell lines may be due to the expression of different proportions of ghrelin receptors; however, the distribution of the alternative ghrelin receptor is currently unknown. Discrepancies in different cell types may also reflect the fact that a wide range of different insults can be applied to promote apoptosis. Further studies are required to determine whether ghrelin may play a role in cancer by stimulating cell proliferation and inhibiting apoptosis, and in vivo studies using mouse xenograft models will help to determine the significance of ghrelin in cancer progression.

B. Ghrelin and cell migration, invasion, and metastasis

The ability of cancer cells to migrate from the primary tumor site, to invade the surrounding tissues, and to spread to distant tissues via hematogenous or lymphatic routes is a hallmark of cancer, and these processes are required for cancer progression (316, 317). Although there have been few studies into the role of ghrelin in these processes, there is some evidence that ghrelin may stimulate cell invasion and migration in a number of cancer cell lines (Table 2). Ghrelin treatment significantly increases cell migration and invasion through Matrigel [a mouse-derived extracellular matrix (ECM)] in the PANC1, MiaPaCa2, BxPC3, and Capan2 pancreatic cancer cell lines (77). Invasion and migration in response to ghrelin treatment occurred through the activation of the phosphoinositide 3 kinase (PI3K)/Akt pathway, a signaling pathway that is often associated with an increase in cell motility and invasion (77). Treatment with D-[Lys-3]-GHRP-6 inhibited ghrelin-stimulated cell invasion and also decreased Akt signaling in these cell lines, indicating that the effects of ghrelin may be mediated by the GHSR1α, which these cells express (77). Alternatively, it is possible that the GHSR1α inverse agonist, D-[Lys-3]-GHRP-6, may also bind and inhibit an alternative ghrelin receptor.

Ghrelin stimulates cell migration in human astrocytoma cell lines, and ghrelin may play an autocrine/paracrine role because these cells also secrete ghrelin (235). In Transwell and scratch assays, ghrelin treatment increased the rate of cell migration and cell invasion through Matrigel in a dose-dependent manner. UAG did not have a significant effect on cell motility, however. Invasion is a cancer-related process that requires the detachment of cells, the degradation of the ECM, and the subsequent migra-
tion of cells through the ECM. The matrix metalloproteases (MMP) are key ECM-degrading enzymes, and their secretion is frequently altered in cancer (156). The enhanced motility and invasiveness exhibited by astrocytoma cells in response to ghrelin treatment corresponded to an increase in MMP-2 activity (235). The effects of ghrelin on MMP-2 expression and cell invasion and motility in astrocytoma cells were abolished when ghrelin secretion was reduced by treatment with small interfering RNA, indicating that these processes are stimulated in an autocrine manner. Ghrelin treatment altered the distribution of the GHSR1a in the cell, increasing its expression at the membrane ruffles at the leading edge of the migrating cell (235). GHSR1a colocalizes with Rac, a member of the Rho family of small GTPases that plays a role in formation of lamellipodia, which are important in cell migration, and this strongly supports a role for GHSR1a in cell migration. Ghrelin signaling occurs through activation of protein kinase C (PKC) in the U-87 cell line, a signaling pathway that has been associated with increased motility and invasion in other cancer cell types. PKC activation and cell motility were inhibited by small interfering RNA treatment down-regulating GHSR1a (235), indicating that these effects were mediated by GHSR1a, rather than through the alternative ghrelin receptor (235). Because ghrelin is also expressed in astrocytoma, it may play a role as an autocrine factor that stimulates cancer progression (235).

Autocrine ghrelin may also stimulate cell invasion in the SW-48 or RKO human colon cancer cell lines because treatment of these cells with a ghrelin-neutralizing antibody, or with the GHSR1a inverse agonist D-[Lys-3]-GHRP-6, significantly decreases the migration and invasion of cells through Matrigel in an invasion chamber (73). Because these effects are blocked by D-[Lys-3]-GHRP-6, they are likely to be mediated through the GHSR1a (73), assuming that this antagonist is specific to the GHSR1a and does not interact with the alternative ghrelin receptor.

C. Ghrelin and angiogenesis

Angiogenesis is a key process in cancer development (317) and is regulated by a balance of proangiogenic and antiangiogenic molecules. For angiogenesis to occur, a number of processes are required, including endothelial cell proliferation, migration, and capillary tubule formation (337). Although the role of ghrelin in angiogenesis associated with cancer has not been investigated, there is evidence that ghrelin has proangiogenic effects in some studies, although antiangiogenic effects have also been reported.

Angiotensin II and fibroblast growth factor 2 (FGF-2) are two major factors that stimulate angiogenesis in health and disease. In human aortic endothelial cells, ghrelin treatment inhibits angiotensin II-stimulated migration, but ghrelin alone had no effect on these cells (337). Ghrelin also inhibits FGF-2-stimulated proliferation and antiapoptotic effects in cultured rat brain endothelial cells and inhibits FGF-2-mediated proliferation and tube formation in human umbilical vein endothelial cells (HUVEC) (338).

In contrast, a number of studies indicate that ghrelin may be proangiogenic, and ghrelin may have different effects on angiogenesis in different vessel types. The human microvascular endothelial cell (HMVEC) line expresses ghrelin and the GHSR1a (339). Ghrelin treatment, at lower concentrations, increases endothelial cell proliferation, whereas concentrations of over 100 nM were inhibitory (339). Ghrelin also stimulates endothelial cell migration, a process that is important in angiogenesis (339). In vitro angiogenesis assays were applied to demonstrate that ghrelin treatment stimulates the formation of capillary-like tubules in Matrigel, signaling through the ERK1/2 pathway. These authors concluded that ghrelin, at physiological concentrations, stimulates angiogenesis, and therefore, ghrelin may have a role in angiogenesis associated with wound healing and may also promote tumor growth by enhancing angiogenesis (339).

A role for ghrelin in promoting angiogenesis is supported by another study demonstrating that a decrease in ghrelin expression is associated with a decrease in angiogenesis in aging HMVEC, and treatment with ghrelin restored angiogenesis through ERK1/2 signaling (340). Ghrelin protects against apoptosis induced in a number of ways and promotes survival in cardiomyocytes and endothelial cells (36, 86, 182, 341). In the H9c2 cardiomyocyte and PAE (pig aortic endothelial) cell lines, ghrelin and UAG protect against apoptosis, with ghrelin signaling through the ERK1/2 and PI3K/Akt pathways (36). These effects are likely to be mediated through the alternative ghrelin receptor because H9c2 cells do not express GHSR1a (36). In diabetes mellitus, advanced glycation end-products are generated and induce apoptosis in endothelial cells. In HUVEC, ghrelin protects endothelial cells from apoptosis induced by advanced glycation end-products by acting through the GHSR1a and stimulating the ERK1/2 MAPK signaling pathway (85).

D. Ghrelin and cancer-related inflammation

Ghrelin has been shown to have an immunomodulatory role and potent anti-inflammatory effects (186, 311), and it could therefore play a role in the prevention of cancers associated with chronic inflammation. Ghrelin is also elevated in patients with cancer cachexia, which has a significant inflammatory component (see Section V). Chronic inflammation and, particularly, elevated levels of IL-6 and IL-1 play a role in tumor progression (342) and may predispose patients to a number of different cancers.
including esophageal, gastric, lung, prostate, and colon cancer, and local inflammation is associated with most tumor microenvironments (343, 344). Antiinflammatory therapies are, therefore, currently of great interest for the prevention or treatment of cancer (344). Ghrelin is expressed in a number of cell types in the immune system, including mast cells, monocytes, macrophages, and dendritic cells (311), and it is likely to be produced locally by a number of cell types in the tumor microenvironment. Cancer-related inflammation has many effects, influencing cell proliferation, survival, angiogenesis, and metastasis, and it may lead to genetic alterations (343).

Ghrelin inhibits the production of proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8 and inhibits the activation of NFκB, a key molecule that regulates inflammation and cytokine production (281, 311). Ghrelin can also up-regulate antiinflammatory molecules, including the cytokine IL-10 (281, 311).

Esophageal cancer is strongly associated with chronic inflammation. In the OE-19 esophageal adenocarcinoma cell line, ghrelin treatment prevents inflammation induced by the potent proinflammatory cytokine TNF-α and decreases the expression of proinflammatory molecules, cyclooxygenase 2 (COX-2) and IL-1β, in this cell line (329). Interestingly, high ghrelin levels were shown to be protective against esophageal cancer, but only in obese subjects (345). A decrease in circulating ghrelin levels in patients with esophageal cancer could down-regulate the protective effect of ghrelin in these cancers. In the A549 NSCLC cell line, ghrelin inhibits the action of the proinflammatory transcription factor, NFκB, and inhibits IL-8 expression stimulated by reactive oxygen species (346). Ghrelin could, therefore, protect the lung against reactive oxygen species, which can play a role in chronic inflammation and the development of lung cancer (347).

Chronic gastric inflammation associated with Helicobacter pylori infection predisposes patients to gastric carcinoma (243). The relationship between blood ghrelin levels and H. pylori infection is unclear, but the development of atrophic gastritis with chronic infection reduces plasma and gastric ghrelin levels due to damage of the gastric mucosa (348). Because ghrelin has protective and antiinflammatory effects on the stomach, lower ghrelin levels in patients with H. pylori could exacerbate this disease, and low ghrelin levels may accelerate changes leading to the development of gastric carcinoma.

Although further studies are required, ghrelin may play a role in inflammatory bowel disease (IBD), and it could be a useful therapeutic agent (321, 349). IBD is a chronic disease that predisposes patients to the development of colorectal cancer. In some studies in rodent models of IBD, ghrelin appeared to have strong antiinflammatory effects (321, 332), and it down-regulated NFκB production, inhibited the production of proinflammatory cytokines, and inhibited the Th1 response (321, 332), with a down-regulation of TNF-α, IL-1β, and IL-6 (321). In a rodent model where IBD was induced using treatment with 2,4,6-trinitrobenzene sulfonic acid (TNBS), colitis led to an increase in the expression of COX-2, and COX-2 expression was greater in animals treated with ghrelin (332). These authors hypothesized that an increase in COX-2 expression in response to ghrelin treatment would lead to an increase in prostaglandin synthesis, which would promote healing in the gut (332). If this finding is replicated, an increase in COX-2 expression could be a concern that could limit the use of ghrelin as a therapeutic in IBD because a number of prostaglandins in the COX-2 pathway promote tumor progression. COX-2 is often elevated in cancer, and COX-2 inhibitors have been shown to have anticarcinogenic activity in colon cancer (350).

In contrast, it has been hypothesized that ghrelin may have proinflammatory effects in colitis, and ghrelin has also been demonstrated to up-regulate NFκB activity and IL-8 expression in human colonic epithelial cells (351). In a mouse model of IBD [induced using 3% dextran sodium sulfate (DSS)], ghrelin treatment worsened symptoms, increased inflammation, and increased levels of the proinflammatory cytokine, IL-1β (352). Interestingly, the disease was less severe in ghrelin knockout mice, and the authors concluded that endogenous ghrelin could exacerbate the response to DSS (352). Although ghrelin ameliorated the effects of colitis in TNBS models and exacerbated disease in a DSS model, the TNBS model is likely to be a better model for human disease, and studies in mice and rats have shown similar responses (349). The majority of studies in a range of model systems have demonstrated that ghrelin largely has antiinflammatory effects (311).

In a rodent TNBS model of IBD, the antiinflammatory effects of ghrelin appear to be mediated by NO, produced by inducible NO synthase, and these effects are antagonized by NO synthase inhibitors (332). Although it has antiinflammatory effects, paradoxically, NO synthesis may also be damaging. It produces reactive nitrogen species, which can cause DNA damage, and this may play a key role in carcinogenesis resulting from chronic inflammation (347). By activating NO, ghrelin may therefore have antiinflammatory effects, but may also lead to increased DNA damage (347).

Hepatocellular carcinoma is associated with chronic inflammation resulting from infection with viral hepatitis (due to hepatitis B and D viruses), and cirrhosis from hepatitis is associated with malnutrition and increased catabolism (353). Serum ghrelin levels are significantly elevated in cases of cirrhosis, and hepatocellular carcinoma is as-
associated with viral hepatitis (353); however, it is unclear whether this is due to the metabolic disturbance associated with cirrhosis or with the cancer itself. TNFα and IL-6 are both elevated in liver disease and ghrelin levels were directly correlated with TNFα levels, although the relationship between plasma ghrelin and TNFα levels remains unclear (353).

Ghrelin appears to have predominantly antiinflammatory effects (311), and we hypothesize that ghrelin could be useful in suppressing the chronic inflammation that is associated with cancer progression. Inflammation may either promote cancer or suppress cancer, however, and antiinflammatory treatments could suppress protective antitumor immunity (342, 344). Inflammation can both promote tumor growth and spread and inhibit tumor progression, and the balance between these effects is poorly understood (344). Cancer immunosurveillance is believed to require, in addition to a number of antiinflammatory cytokines, the expression of proinflammatory cytokines, including IL-1α, IL-1β, and IL-6 (342). In some models of inflammation, ghrelin has been demonstrated to suppress IL-1β or IL-6 (281, 329, 349). A better understanding of the influence of ghrelin on cancer immunosurveillance, tumor promotion, and tumor-preventing effects is required to determine whether ghrelin may promote or prevent cancer progression through its antiinflammatory actions.

V. Ghrelin as a Treatment for Cancer Cachexia

Cachexia is a complex, multifactorial process associated with increased inflammation, catabolism, impaired energy balance, anorexia, muscle wasting, lipolysis, and substantial weight loss (104, 311, 354). Cachexia is associated with a number of chronic diseases including congestive heart failure, chronic renal failure, AIDS, chronic obstructive pulmonary disease, and cancer, and it may occur as a result of cancer chemotherapy (104, 354). Cancer cachexia is frequently associated with malignancy and contributes to an increased cancer mortality rate (355). Cachexia also reduces the quality of life of patients, who frequently experience nausea and fatigue (355), and new treatments to more effectively manage the symptoms are urgently required. Plasma ghrelin levels are elevated in cancer cachexia (see Section III.D), and although the mechanisms leading to elevated ghrelin levels in cachexia are unclear, ghrelin may be secreted in response to negative energy balance, reduced food intake, and inflammation associated with cachexia (311). Treatment with ghrelin, ghrelin analogs, and GHS may be useful as an approach for cancer cachexia and cachexia resulting from other diseases (354).

Ghrelin and synthetic GHS could ameliorate a number of the symptoms of cancer cachexia through a range of mechanisms, including its antiinflammatory effects and its ability to promote appetite stimulation, improve gastric motility, and alter energy balance. The orexigenic effects of ghrelin may be particularly beneficial (356), and better treatments for cancer-related anorexia, with fewer side effects than traditional treatments, are urgently required. In healthy male and female volunteers, ghrelin treatment stimulates food intake by approximately 28%, and no adverse side effects have been observed (5).

Ghrelin has been shown to be beneficial in mouse models of cancer cachexia. In cachectic MCG101 mice bearing methylcholanthrene-induced sarcoma cells, the animals demonstrated anorexia, fat loss, and muscle wasting and elevated levels of proinflammatory cytokines, TNFα, IL-1β, and IL-6 (325). Higher doses of ghrelin (given ip twice daily) improved food intake and body composition, including increased body weight and fat mass in mice with tumors, compared with mice that were not treated with ghrelin or mice that received lower doses (325). Ghrelin treatment increased the expression of hypothalamic GH/1a and plasma ghrelin levels in these mice, however, which indicates that some ghrelin resistance had developed (325). Because ghrelin levels are elevated in cancer cachexia, supraphysiological doses of ghrelin are likely to be required to exert a positive effect on appetite and body composition in patients with cachexia (326). Using a mouse model of cancer cachexia, in nude mice bearing a human SEKI melanoma cell line xenograft, twice daily ghrelin injections over 6 d increased food intake and decreased weight loss compared with controls (357, 358). Ghrelin treatment did not stimulate feeding in a rat cachexia model, however, where anorexia was induced by the sc implantation of methylcholanthrene-induced sarcomas (359). In this model, ghrelin infusion led to a significant maintenance of fat mass compared with untreated controls, but muscle protein was not preserved (359). This study indicated that ghrelin may not be useful for treating anorexia in cancer cachexia, although a number of studies have demonstrated a beneficial effect.

In a small study of cancer patients with anorexia, the acute infusion of ghrelin for 90 min led to a significant increase in caloric content ingested and improved subjective scores of meal appreciation, with treatment stimulating appetite in all of the subjects studied (360). In another small study comparing the effects of daily high-dose ghrelin (10 μg/kg) and low-dose ghrelin (0.5 μg/kg) in patients with malignant gastrointestinal disease, weight loss, and anorexia, patients in the high-dose group demonstrated
better subjective appetite scores, serum GH, and improved whole body fat retention compared with the low-dose group (356). Although there were many limitations to this study, ghrelin treatment did not cause any significant side effects and did not alter the expression of tumor-related biomarkers, providing some evidence that ghrelin did not accelerate cancer progression in this short-term study (356).

GHSR1a agonists are likely to be useful substitutes for ghrelin in the treatment of cancer cachexia because ghrelin has a very short half-life, requiring iv infusions (326). In a tumor-bearing rat model of cancer cachexia, animals were treated with ghrelin or the GHSR1a agonist BIM-28131 (Ipsen, Milford, MA), which helped to maintain lean body mass (326). This, therefore, has potential as a therapeutic intervention for cancer cachexia (326). The ghrelin mimetic, RC-1291 (Helsinn Therapeutics, Bridgewater, NJ) is an orally active GHS with potential as a treatment for cancer cachexia (361). In healthy patients, RC-1291 stimulates a significant increase in appetite, food consumption, and weight gain and was well tolerated (361), and in phase II trials in cancer patients, treatment improved total body mass, lean body mass, and hand-grip strength (361).

Because the pathophysiology of cachexia has a significant inflammatory component, some of the effects of ghrelin in cachexia may result from its antiinflammatory properties; however, this has not yet been proven experimentally. Treatment of tumor-bearing rats with ghrelin, or BIM-28131, reduced the expression of hypothalamic IL-1 receptor, and this is likely to reduce the response to IL-1β and may play a role in mediating the anticytotoxic activity of ghrelin (326). In this study, ghrelin and BIM-28131 treatment did not alter plasma cytokine expression, however (326). In patients with colon cancer and elevated levels of proinflammatory cytokines, ghrelin levels were elevated compared with plasma levels in normal controls (313).

Chemotherapy for cancer can also lead to significant side effects including anorexia, weight loss, nausea, vomiting, and disrupted gut motility, and it decreases the quality of life of cancer patients (362). In patients with cachexia associated with lung cancer, ghrelin levels were elevated in patients with anorexia after chemotherapy, but ghrelin levels were not altered in patients without anorexia and decreased food intake (159). Cisplatin is a commonly used chemotherapeutic, with significant side effects, including nausea, vomiting, and anorexia and a decreased quality of life (363). A recent study in cisplatin-treated rats demonstrated that treatment decreased hypothalamic ghrelin expression, and this may contribute to anorexia associated with this drug (363). In a cisplatin chemotherapy-associated dyspepsia model in rodents, the ip injection of ghrelin improved feeding and energy intake and led to an increase in locomotor activity, which may indicate a reduction in the level of nausea experienced (362). Ghrelin, injected ip, is believed to interact with GHSR expressed by vagal nerve endings, and these vagal afferents communicate with nuclei in the hypothalamus (362). Because rodents are incapable of vomiting, it could not be demonstrated that ghrelin acted as an antiemetic in this model; however, a similar study in ferrets demonstrated that ghrelin reduced vomiting induced by cisplatin treatment when it was injected into the cerebral ventricles (364).

Studies that have demonstrated that ghrelin treatment increases cell proliferation (33, 36, 41–66, 69, 70, 73–77, 127), protects against apoptosis (36, 54, 57, 60, 63–67, 69, 72, 74, 82–93), or increases cell migration or invasion (73, 77, 235, 339, 365) may indicate that there should be some caution associated with the use of ghrelin for cancer cachexia and for treating symptoms associated with chemotherapy, particularly because supraphysiological doses of ghrelin are likely to be required. A number of short-term studies have demonstrated that ghrelin treatment does not increase tumor size or the expression of tumor markers (325, 326, 366), but elevated ghrelin levels led to gut hypertrophy and gastric tumors in transgenic mice and stimulated the IGF-I axis, which is strongly linked with cancer (305, 306). Longer term in vivo studies are required to determine whether ghrelin could promote tumor growth, particularly in cancer patients.

VI. Obestatin and Cancer

Obestatin plays a role in cell proliferation, and there is some evidence that it could have a role in cancer progression. Obestatin is expressed in a number of normal tissues, including the gastrointestinal tract, with highest levels in the stomach and lower levels in the duodenum and colon (98). Obestatin expression has also been demonstrated immunohistochemically and at the mRNA level in the anterior pituitary, pancreatic islets, and bronchi (98), and it is also expressed in the liver, thyroid gland, testes, and mammary glands (98, 367–369). In the pancreatic islets, obestatin colocalizes with ghrelin expression, and in the anterior pituitary it colocalizes in the somatotrophs with GH (98). In normal fetal tissues, obestatin is expressed in the thyroid gland, pituitary, lung, pancreatic islets, and gastrointestinal tract, whereas most other tissues exhibit no obestatin immunoreactivity (98).

Obestatin expression has been demonstrated immunohistochemically in a relatively small proportion of endocrine tumors, including thyroid carcinomas (papillary, fol-
licular, and medullary) (98, 370), in well-differentiated neuroendocrine tumors of the stomach and small intestine, and some neuroendocrine pancreatic tumors (98). The obestatin peptide is expressed in normal stratified squamous epithelium, but expression is reduced in oral squamous cell carcinoma, and levels are lowest in the most poorly differentiated cancers (371). Plasma obestatin levels are elevated in patients with ovarian cancer and benign ovarian tumors (289). It is currently unclear whether the tumors themselves are the source of elevated plasma obestatin levels; however, obestatin levels could be a useful marker for ovarian tumors. In contrast, in patients with uterine leiomyoma and prostate cancer, obestatin plasma levels were not elevated (277, 278).

In elderly men with chronic atrophic gastritis, but without H. pylori infections, obestatin levels are significantly lower than in healthy controls, and the preprandial plasma ghrelin to obestatin ratio is also reduced (367). As is the case with ghrelin, obestatin levels are likely to be decreased as a result of damage to ghrelin-secreting cells in the gastric mucosa (367). H. pylori infection plays a key role in the development of many gastric carcinomas (372). In a study in Chinese subjects, obestatin levels were not different between patients with and without H. pylori infections, but the obestatin/ghrelin ratio was lower in patients with H. pylori and was inversely correlated with BMI (372). Ghrelin and obestatin could play a role in the pathogenesis of atrophic gastritis and H. pylori infection (372).

Although there have been few studies into the role of obestatin in cancer, it may play a role in a number of processes related to cancer progression, and it influences cell proliferation in some normal-derived cell lines. In the INS-IE and HIT-T15 pancreatic β-cell lines and in isolated human pancreatic islet cells, obestatin stimulates cell proliferation (94). In primary cultures of retinal pigment epithelial cells, exogenous obestatin stimulates cell proliferation through activation of the ERK1/2 MAPK pathway (95). Obestatin signaling in these cells appeared to be mediated through the G protein-coupled receptor subunit, and activation of PI3K, PKC, and Src (95). Obestatin could play a role in diseases associated with uncontrolled retinal cell proliferation, including proliferative vitreoretinopathy, where epithelial cells migrate, differentiate into myofibroblasts, and then proliferate (95).

There have been few studies into the role of obestatin in cancer cell proliferation. In the KATO-III gastric cancer cell line, exogenous obestatin treatment stimulates cell proliferation, but obestatin had no effect on cell proliferation in the A549 normal stomach-derived cell line (97, 236). Obestatin stimulated cell proliferation in the KATO-III cell line through activation of the MAPK, ERK1/2 pathway, and this signaling appears to be mediated by PKCe phosphorylation and activation of a pertussis toxin-sensitive G protein (97). Similar results were observed in human retinal pigment epithelial cells, and together, these studies suggest that obestatin signals in these cells through a GPCR, the identity of which remains unknown (97). Further dissection of the obestatin signaling pathway in the KATO III gastric cancer cell line indicated that obestatin may signal through cross talk with the epidermal growth factor receptor (EGFR) (373). Although it is largely accepted that GPR39 is not the obestatin receptor (21), this study suggested that obestatin stimulates the formation of a complex between GPR39, β-arrestin, and the signaling molecule Src (373). This resulted in transactivation of the EGFR, which required MMP activity, but did not require pertussis toxin-sensitive G proteins or the GBy-subunit (373). EGFR activation stimulated the phosphorylation of Akt, which required the activation of phosphoinositol-dependent kinase and mammalian target of rapamycin kinase complex 2 (373). This potential role of GPR39 in obestatin signaling requires further clarification, however.

Using cDNA microarray studies, it was demonstrated that GPR39 was up-regulated in primary esophageal squamous cell carcinomas (ESCC) compared with matched normal controls (374). GPR39 protein expression was elevated in over 50% of 207 ESCC tested compared with controls, and expression was associated with lymph node metastasis and advanced disease (374). Forced overexpression of GPR39 increased the rate of cell proliferation, clonogenic growth, and cell migration and invasion in ESCC cell lines. The morphology of the cells was altered toward a fibroblastic phenotype from a more cobblestone appearance, and cells showed signs of lamellipodia formation and cytoskeletal rearrangements, indicating an increase in cell motility (374). These cells formed larger tumors in xenografts in nude mice, and GPR39 could, therefore, be a useful therapeutic target in ESCC that overexpress this receptor (374).

In some cell lines, exogenous obestatin treatment appears to decrease cell proliferation. Obestatin is secreted into the culture medium at levels higher than human serum from the BON-1 pancreatic neuroendocrine and the TT thyroid carcinoma cell lines (98). At high concentrations, obestatin treatment decreased cell proliferation in these cell lines, in contrast to ghrelin, which increases cell proliferation at similar concentrations (98). In the mouse ATDC-5 embryonic carcinoma-derived cell line, which has been differentiated into chondrocytes, obestatin inhibits cell proliferation, and it has a similar effect in the C28–12 normal human rib-derived chondrocyte cell line (99).

Although the role of obestatin in apoptosis in cancer has not been investigated, obestatin protects against ap-
optosis in normal cell types and cell lines. In the rodent INS-IE and HIT-T15 pancreatic β-cell lines and in isolated human pancreatic islet cells, obestatin enhances cell survival and protects against apoptosis stimulated by serum withdrawal, or by treatment with proinflammatory cytokines, by stimulating PI3K/Akt and ERK1/2 pathways (94). In these cells, obestatin up-regulates the expression of genes associated with cell survival (94). In rat H9c2 cardiac cells, isolated myocardiocytes, and a rat isolated heart model of ischemia/reperfusion injury, obestatin protected against apoptosis through activation of Akt, PI3K, PKCe, PKCδ, and ERK1/2 (375). Obestatin treatment stimulates porcine ovarian granulosa cell proliferation, and in contrast to other cell lines, it also stimulates apoptosis, indicated by increased expression of caspase 3 and Bax (376). Obestatin may play a role in protecting these tissues against a number of conditions that stimulate apoptosis and could also increase cell survival in cancer cells, promoting cancer progression.

The role of obestatin in cancer-related inflammation has not been investigated; however, obestatin may also have antiinflammatory properties. In a cerulein-induced pancreatitis rat model, obestatin protected against the development of acute pancreatitis, improved blood flow to the pancreas, and reduced plasma levels of the key proinflammatory molecule, IL-18 (377). Although cerulein treatment decreased cell proliferation (DNA synthesis) in pancreatic cells, obestatin treatment partially reversed this effect (377).

VII. Summary and Perspectives

Ghrelin, GHSR1a, and obestatin are widely expressed in normal tissues, and ghrelin is expressed in many cancer and tumor cell types and cell lines (Table 1). Ghrelin expression may be up-regulated in some cancers, including colon and breast cancer (73, 242). Although the pathophysiological significance of this expression is unclear, there is evidence that ghrelin could be useful as prognostic or diagnostic markers for prostate cancer in particular (256) and possibly for other cancers (43, 246). In vitro studies using a fluorescein-labeled, C-terminally truncated 18-amino acid ghrelin probe demonstrated that the quantification of binding to ghrelin receptors in prostate tissues allows prostate cancer to be distinguished from BPH and normal prostate (256). Because better prognostic and diagnostic methods for prostate cancer are urgently required, this method, coupled with in vivo imaging techniques, could provide an exciting and powerful new tool and could be used to guide surgery and spare normal tissues. There is some evidence that the ghrelin axis could also be useful as a marker for breast cancer. A novel, high-frequency methylation of the GHSR promoter region enabled researchers to distinguish between invasive breast cancer and normal breast tissues with high specificity and sensitivity (274). The functional significance of the down-regulation of GHSR1a that is observed in some cancers is unknown.

Although no GWAS have linked the ghrelin axis with cancer, some SNP in the ghrelin or GHSR genes have been associated with an increased risk of breast cancer (100) and esophageal cancer (264, 265) and a decreased risk of colorectal cancer (103) and non-Hodgkin’s lymphoma (101). Some small studies have shown no associations in a number of cancers (260, 262, 263). Due to the molecular heterogeneity of diseases such as breast and prostate cancer, larger studies and more patient stratification according to disease phenotype are likely to be required to determine whether the ghrelin axis plays a role in these cancers.

Serum ghrelin is unlikely to be a useful biomarker for the diagnosis of cancer because ghrelin levels are closely correlated with BMI. It may provide a useful early marker for cancer cachexia, however, particularly in NSCLC (281). Elevated ghrelin levels associated with a decrease in BMI are associated with cancer cachexia (73, 159, 280, 281). Although plasma ghrelin levels have been shown to be elevated in some cancers (240, 277, 278, 281, 289, 303, 304), many studies need to be interpreted with caution because there are a number of limitations in the measurement of acylated ghrelin. A number of studies have been performed where the rapid deoctanoylation and degradation of ghrelin is not prevented, and these measurements may be inaccurate (114, 193, 287). In addition, results vary greatly between assays, and there are no international standards (283). The introduction of two-site sandwich ELISA has improved the sensitivity and specificity of acylated ghrelin assays (283). High throughput and sensitive quantitative mass spectrometry assays (293) are likely to provide very accurate measurement of both acylated and unacylated ghrelin and their degradation products, but these assays also require careful sample preparation.

Ghrelin stimulates cell proliferation in the majority of normal cell types and cell lines tested to date (33, 36, 44–66), and therefore, it is not surprising that ghrelin also stimulates cell proliferation in a range of cancer and tumor cell types (41–43, 69–77, 79), although it has been reported to inhibit proliferation in others (34, 80, 81) (Table 2). The response to ghrelin is likely to be cell type-specific, however, and differences could reflect the differences in the expression of GHSR1a and the hypothesized alternative ghrelin receptor. Findings by different research groups that ghrelin can stimulate or inhibit proliferation in the
same cell lines may result from differences in the cell lines themselves, a difference in the methods used to perform the assays, or the concentrations and purity of ghrelin used. In vitro studies into the action of acylated ghrelin in particular would require the frequent replenishment of ghrelin because the peptide is rapidly degraded and the octanoyl group rapidly removed (114, 193, 287). This could alter the effects of treatment that are observed. The physiological significance of the concentrations of ghrelin used in these assays is difficult to determine because tumor levels of ghrelin have not been measured.

Ghrelin may also influence cell number by regulating apoptosis and cell survival (Table 2). Ghrelin has been shown to protect against apoptosis induced by a number of different insults in the nervous system, cardiovascular system, pancreas, and a range of other normal and cancer or tumor cell types (36, 54, 57, 60, 63–67, 69, 72, 74, 82–93), but it has been shown to increase the rate of basal or induced apoptosis in some cell lines (79, 80, 328).

There have been few studies investigating the role of ghrelin in cell invasion and migration, two processes that are critical for cancer progression and metastasis. Ghrelin treatment has been shown to increase cell migration and invasion in colon cancer, astrocytoma, and pancreatic cancer (73, 77, 235), and it increases migration in normal microvascular endothelial cells (339, 365). Ghrelin has been shown to have a role in regulating processes required for angiogenesis. Ghrelin increases angiogenesis, stimulating cell proliferation or migration and inhibiting apoptosis in most endothelial cell types studied (36, 56, 85–87, 182, 339, 365). It has also been shown to inhibit processes required for angiogenesis in some studies, however, and it inhibits the angiogenic response to FGF-2 (337, 338, 379). The role of ghrelin in angiogenesis associated with cancer has not been investigated, however, although it has been hypothesized that ghrelin could promote wound healing and tumor growth by stimulating angiogenesis (339).

In the few studies that have investigated the role of ghrelin in cancer progression in rodent models of cachexia, no increases in cell number have been observed in short-term studies (325, 326), and ghrelin did not appear to accelerate cancer progression in a small short-term study of patients with gastrointestinal cancer (356). In contrast, transgenic mice overexpressing ghrelin developed hypertrophy of the gut, ghrelin-producing tumors, and elevated IGF-I levels (305). Elevated IGF-I levels are associated with the development of cancer (306). Further, longer term, and more targeted in vivo studies are required to determine whether changes in cell proliferation are pathophysiologically significant.

It is possible that the ghrelin axis could be a useful target for adjunct therapies for some cancers, where ghrelin promotes processes related to cancer progression, including the stimulation of cell proliferation, migration, and invasion and the inhibition of apoptosis. It is also feasible that ghrelin antagonists may have similar effects to GHRH antagonists. Interestingly, the classical GHRH has been demonstrated to have autocrine/paracrine actions in the prostate (380–383), and GHRH antagonists (synthetic GHRH analogs) are promising treatments for BPH, castrate-resistant prostate cancer, and other cancers including breast cancer and colorectal cancer (381, 384). GHRH is a GHSR1a agonist, which binds the receptor and stimulates calcium signaling, and some GHRH receptor antagonists have also been demonstrated to bind the GHSR1a (385).

Ghrelin has been shown to have largely antiinflammatory effects, and it protects cells against a number of insults (311), although proinflammatory effects have been described in some studies (351, 352). Because chronic inflammation is a risk factor for the development of some cancers, including gastric, esophageal, lung, prostate, and colon cancer (343), we hypothesize that ghrelin may protect against the development of some cancers. Because inflammation can promote or inhibit cancer (344), it is difficult to predict the effect of ghrelin treatment on tumor growth, and specific studies into the role of ghrelin in cancer-related inflammation are required. The antiinflammatory effects of ghrelin, in addition to its influence on feeding and energy balance, appear to be of benefit to patients with cancer cachexia (104, 354). Ghrelin and its analogs show promise as a treatment for cancer cachexia, which is associated with inflammation, muscle wasting, weight loss, anorexia, and decreased quality of life. Although the role of ghrelin in cancer progression remains unclear, the benefits of this treatment are likely to outweigh the potential risks in these patients.

Ghrelin is a multifunctional hormone with roles in regulating a number of processes related to cancer progression, including cell proliferation, apoptosis, cell invasion and migration, and angiogenesis. In particular, the finding that a fluorescently labeled ghrelin analog binds prostate tissues and can be used to discriminate between benign prostate disease and prostate cancer indicates that the ghrelin axis may be important in prostate cancer and further studies are warranted. It is currently unclear whether the ghrelin axis has tumor-promoting effects, or indeed whether it may inhibit tumorigenesis in vivo, and further studies are therefore required to elucidate its role in cancer.
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