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*Review*

# Roles for neuregulins in human cancer

Christophe Stove[1,2] & Marc Bracke[1]
[1]*Laboratory of Experimental Cancerology, Department of Radiotherapy and Nuclear Medicine, Ghent University Hospital, Ghent, Belgium;* [2]*Units of Molecular Cell Biology and Molecular and Cellular Oncology, Department for Molecular Biomedical Research, VIB-Ghent University, Zwijnaarde, Belgium*

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**Abstract**

The human epidermal growth factor (EGF) receptor (HER) family of receptor tyrosine kinases has frequently been implicated in cancer. Apart from overexpression or mutation of these receptors, also the aberrant autocrine or paracrine activation of HERs by EGF-like ligands may be important in cancer progression. Neuregulins constitute a family of EGF-like ligands that bind to HER3 or HER4, preferably forming heterodimers with the orphan receptor HER2. Mesenchymal neuregulin typically serves as a pro-survival and pro-differentiation signal for adjacent epithelia. Disruption of the balance between proliferation and differentiation, because of autocrine production by the epithelial cells, increased sensitivity to paracrine signals or disruption of the spatial organization, may lead to constitutive receptor activation, in the absence of receptor overexpression. Consequently, the analysis of ligand expression and/or activated receptors in tumor samples may broaden the group of patients that can benefit from targeted therapies.

*Abbreviations:* AMF – autocrine motility factor; ARIA – acetylcholine receptor-inducing activity; ATF4 – activating transcription factor 4; COX-2 – cyclooxygenase-2; EGF – epidermal growth factor; E2 – estradiol; EF-1$\alpha$ – elongation factor-1$\alpha$; ER – estrogen receptor; FAK – focal adhesion kinase; G3BP – GTPase-activating protein SH3 domain-binding protein; GADD153 – growth-arrest and DNA-damage 153; GAP – GTPase-activating protein; GGF – glial growth factor; HER – human EGF-like receptor; HIF-1$\alpha$ – hypoxia inducible factor-1$\alpha$; hnRNP K – heterogeneous nuclear ribonucleoprotein K; HRG – heregulin; HSP70 – heat shock protein-70; I$\kappa$B – inhibitor of $\kappa$B; MAPK/ERK – mitogen-activated protein kinase/extracellular-regulated kinase; MMP – matrix metalloproteinase; MMTV – murine mammary tumor virus; MTA1 – metastasis and tumor-associated protein 1; NDF – neu differentiation factor; NF$\kappa$B – nuclear factor $\kappa$B; NRG – neuregulin; Pak1 – p21-activated kinase 1; PGE2 – prostaglandin E2; PI3K – phosphoinositide 3-kinase; PKB/Akt – protein kinase B; PR – progesterone receptor; RAFTK – related adhesion focal tyrosine kinase; RAR$\alpha$ – retinoic acid receptor $\alpha$; SMDF – sensory and motor neuron-derived factor; uPA(R) – urokinase plasminogen activator (receptor); VEGF – vascular endothelial growth factor

## The human epidermal growth factor receptor family

On the basis of their sequence homology and structural characteristics, growth factor receptors can be classified into different groups. The human epidermal growth factor (EGF) receptor (HER) family of receptor tyrosine kinases consists of four members: HER1 (EGFR, ERBB1), HER2 (neu, ERBB2), HER3 (ERBB3) and HER4 (ERBB4). They are widely expressed in epithelial, mesenchymal and neuronal tissues, playing fundamental roles during development and being implicated in the regulation of a variety of biological processes, including cell proliferation, apoptosis and differentiation [1, 2]. Structurally, they consist of a ligand-binding extracellular domain, a single transmembrane domain and an intracellular tail. The latter contains the kinase domain and multiple tyrosine residues that become phosphorylated upon activation and may serve as docking sites for signal transduction molecules. Although all family members share considerable homology, two members deviate from this general description: the extracellular domain of HER2 has not been shown to be involved in ligand binding (orphan receptor) and the HER3 intracellular tail is devoid of kinase activity (kinase dead receptor) [3, 4]. This receptor family has been implicated frequently in human malignancies. This is particularly the case for

*Correspondence to:* Marc Bracke, Units of Molecular Cell Biology and Molecular and Cellular Oncology, Department for Molecular Biomedical Research, VIB-Ghent University, Technologiepark 927, 9052 Zwijnaarde, Belgium. Tel: +32-9-240-3007; Fax: +32-9-240-4991; E-mail: brackemarc@hotmail.com

HER1 and HER2. HER2 overexpression has been described in a wide variety of cancer types (e.g. breast and ovarian) and correlates with poor prognosis [5, 6]. Overexpression of this receptor leads to ligand-independent homodimerization, resulting in constitutive receptor activation, sufficient to induce transformation. Alternative mechanisms leading to ligand-independent constitutive HER2 signaling include a point mutation in the transmembrane domain (only found in rodents) and truncation of non-catalytic sequences [7].

## Neuregulins

Based on distinct biological activities, neuregulins were initially purified as related factors released by a variety of cell types, and were subsequently given different names (NDF, HRG, GGF, ARIA), leading to a confusing nomenclature. Using HER2 activating potential as a read-out, Peles et al. and Wen et al. identified neu differentiation factor (NDF) as a factor released by H-Ras-transformed rat fibroblasts [8, 9], whereas Holmes et al. isolated heregulin (HRG) from medium conditioned by the MDA-MB-231 human mammary cancer cell line [10]. Other groups used neuronal tissue extracts as a source for protein purification: glial growth factor (GGF) was purified from bovine pituitary glands as a Schwann cell mitogen [11] and acetylcholine receptor inducing activity (ARIA) was purified from chicken brain extracts [12]. All these factors, identified in different species, are derived from alternative splicing of a single gene, *neuregulin-1* (NRG-1) [11, 13]. Although it was initially thought that NRG-1 would directly bind and activate HER2, the observation that certain cell lines which express a functional HER2 did not respond to NRG, indicated that another component was needed to mediate the tyrosine phosphorylation of HER2. This was confirmed by the cloning of HER3 and HER4 and their subsequent identification as the primary NRG-binding receptors [14–16].

NRG-1 isoforms are widely expressed in numerous tissues, including brain, heart, skeletal muscle, breast, liver and lung [17, 18]. Based on sequence homology with NRG-1, neuregulin-2 (also termed divergent of neuregulin-1 (Don-1) or neural- and thymus-derived activator of ErbB kinases, NTAK), was cloned [19–21]. Later, two additional neuregulin families (neuregulins-3 and -4) were identified [22, 23]. The latter two only induce activation of HER4, whereas NRGs-1 and -2 bind and activate both HER3 and HER4.

## Structural features of neuregulin-1

At present, at least 26 different neuregulin-1 isoforms have been identified in different species. Ten of these were identified in human, all resulting from alternative splicing of a single gene, located in the short arm of chromosome 8 [24] (Figure 1). Based on the N-terminal domain, they are subdivided into three types: HRG (type I) [10], GGF (type II) [11] and sensory and motor neuron derived factor (SMDF, type III) [25] [reviewed in 13]. Although the N-terminal domain of all three types is involved in cell–surface association, it does so in different ways. HRG and GGF can be distinguished from SMDF by the presence of a heparin-binding immunoglobulin-like sequence in their N-terminal domain. This feature was shown to improve receptor binding by association with cell surface heparin sulphate binding proteoglycans, and may function to sequester these factors in the extracellular matrix as well [26]. Instead, the N-terminal domain of SMDF contains an additional hydrophobic domain that may function as a transmembrane sequence. Insertion of this sequence into the membrane results in the formation of a type II transmembrane protein, with the receptor-activating domain exposed [27–29]. The only exon that is shared by all isoforms is the one encoding the major part of the EGF-like domain. It encodes five of the six cysteines present in the EGF-like domain, the sixth encompassed in the sequence encoded by either the $\alpha$- or $\beta$-exon. This EGF-like domain is both necessary and sufficient for receptor activation, with $\beta$-isoforms having higher receptor affinity and generally being more potent than $\alpha$-isoforms [30].

In transmembrane isoforms, the EGF-like domain is connected to the transmembrane domain by a juxtamembrane domain of variable length ($\alpha/\beta$ 1 or 2), which is susceptible to proteolytic cleavage, releasing the ectodomain [30, 31]. Alternatively, read-through of the $\beta$-encoding exon results in isoforms lacking the transmembrane domain, which are presumably retained intracellularly. Further alternative splicing leads to different lengths of intracellular tails, a, b and c, the a- and c-tail being the longest and shortest, respectively [32]. The role of this intracellular tail is mainly unknown. The two interacting molecules that have been identified are both zinc finger proteins: LIM kinase 1 [33], a serine/threonine kinase that blocks the actin depolymerizing activity of cofilin and has been implicated in cancer cell invasion [34], and a promyelocytic leukemia zinc finger-related protein of undetermined function [35]. However, the functional relevance of these interactions has not been established, yet. In addition, the cytoplasmic tail was shown to be necessary for the apoptosis-inducing properties of neuregulins (see below) and may play a role in 'reverse' signaling. The latter occurs following binding of the extracellular domain of the HRG type III precursor to HER2/4 dimers (reverse juxtacrine signaling) and involves the $\gamma$-secretase-mediated proteolytic release of the HRG intracellular domain and its subsequent nuclear translocation, where it may influence the transcription of target genes [35]. This nuclear translocation was dependent on the presence of a nuclear localization sequence in the HRG juxtamembrane domain [35] and has been observed in several types of

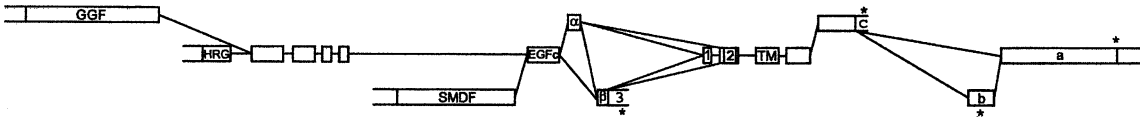


*Figure 1.* Schematic representation of the exon organization and possible alternative splicing of the NRG1 gene. Closed boxes indicate exons (on scale), lines between these indicate possible splicing events, open boxes represent untranslated regions, which may be of varying lengths, depending on the isoform. Introns, which may be very large, are not on scale. An asterisk indicates the position of stop codons. Three different start codons may be used, generating glial growth factor (GGF), heregulin (HRG) or sensory and motor neuron derived factor (SMDF). The epidermal growth factor (EGF)-like domain is composed of a common exon and an $\alpha$ or $\beta$ exon. A combination of an $\alpha$- and $\beta$-exon, found infrequently, leads to the generation of a premature stop codon within the $\beta$ exon (not indicated). Read-through of the $\beta$-exon leads to the generation of a stop codon, while further splicing generates $\alpha/\beta$-1 or -2 isoforms. Following the sequence encoding the transmembrane (TM) domain, alternative splicing leads to isoforms having a-, b- or c-type intracellular tails.

cancer [36–38] Other roles for the transmembrane/cytoplasmic domain include the regulation of the proteolytic release of mature HRG [39–41] and the correct trafficking and subcellular targeting of neuregulins to e.g. rafts, specialized membrane platforms rich in cholesterol [32, 42, 43].

Transmembrane heregulins (with an a-tail) are synthesized as transmembrane precursor proteins of $\pm$ 105 kDa. Following cleavage in the juxtamembrane domain by metalloproteinases [44, 45], they are released as $\pm$45 kDa secreted factors that may bind to nearby receptors and thus function as autocrine/paracrine factors [42, 31]. Alternatively, intact transmembrane HRG molecules may activate HERs, leading to juxtacrine signaling [46], which may be bi-directional: 'forward', leading to HER activation and 'reverse', leading to modulation of intracellular pathways by the HRG cytoplasmic tail, as described above.

Based upon its expression *in vivo*, heregulin is often referred to as a neuronal ($\beta$-isoforms) or mesenchymal ($\alpha$-isoforms) factor [17, 18, 32]. In the latter case, it has been suggested to function as a paracrine factor for the neighboring cells, such as glia, muscle or epithelia, which express HER3 and/or HER4 [17]. The importance of such paracrine signaling is exemplified in *NRG-1* knockout mice, where targeting of the EGF-like domain results in disruption of all NRG-1 isoforms. These mice die *in utero* at embryonic day 10.5 due to a lack of trabeculation, a developmental process involved in ventricular differentiation during heart formation. This process requires the activation of ErbB2 and ErbB4 in the myocytes by HRG, which is normally released from the nearby endocardium [47].

## Signaling induced by neuregulins

Based upon the recent elucidation of the crystal structures of HER1, HER2 and HER3 [48–53], a unique model has been proposed for HRG-induced receptor activation. In this model, binding of HRG to HER3 leads to a ligand-driven conformational change of HER3, allowing a back-to-back association with HER2 [reviewed in 54]. On its turn, HER2 will auto-phosphorylate and trans-phosphorylate HER3, resulting in the generation of multiple docking sites for signal transduction molecules in their cytoplasmic tails [reviewed in 1]. Thus, this represents a unique system in which an orphan receptor (HER2) and a kinase-dead receptor (HER3) cooperate to generate a potent signal [reviewed in 55]. Following HER2 activation, many phosphotyrosines in its carboxy-terminal tail serve as docking sites for Src homology-2- and phosphotyrosine-binding-domain containing proteins, such as Shc, Grb2 and Grb7, generating a highway leading to mitogen activated protein kinase/extracellular regulated kinase (MAPK/ERK) activation [56]. Activated HER3, in contrast, harbors multiple docking sites for the p85 subunit of phosphoinositide 3-kinase PI3K [57]. Thus, it is not surprising that the combined activation of both HER2 and HER3 by HRG provides the cells with a very potent signal. In addition to the 'classical' MAPK/ERK and PI3K pathways, multiple other pathways have been shown to be activated by HRG. Examples include the p38MAPK, ERK5, protein kinase C, phospholipase C$\gamma$ and signal transducer and activator of transcription (STAT) pathways. These have been reviewed recently and will not be discussed into detail here [55, 58, 59]. In addition, HRG-mediated activation of HERs may result in the nuclear localization of full-length or cleaved receptors, suggesting direct, receptor-mediated signaling [60, 61]. Moreover, adding an additional level of complexity to the system, also trans-activation of other receptors may contribute to HRG's actions [58]. For example, HRG has been shown to trans-activate the progesterone receptor, in a HER2- and MAPK-dependent way, leading to enhanced proliferation of mammary cancer cells [62]. The combined action of all or of several of these pathways on transcriptional and post-transcriptional (mRNA stability, translation, post-translational modifications, and protein stability) events determines the final outcome in a given system.

In cancer, aberrant receptor activation may be the result of truncation, mutation, association with other cell-surface proteins, transactivation via other receptors or the presence of autocrine loops [7, 63]. Recently, an interesting physiological control

mechanism for the HRG-HER system was described in human differentiated airway epithelia, in which both HRG-α and its receptors are expressed. In these epithelia, HRG, which is apically expressed, is physically segregated from the basolaterally expressed receptors, preventing HER activation. However, upon disruption of the integrity of the tight junctions, HRG may locally activate its receptors [64]. Thus, this model suggests an additional mechanism for abnormal receptor activation in diseases, such as inflammation and cancer, in which increased epithelial permeability occurs.

Although the exact mechanism(s) by which NRG-1 may lead cells towards malignancy may be distinct for different cell types, some general aspects hold for the majority of these. Cancer cells that aberrantly produce NRG-1 are likely to use it in an autocrine manner, resulting in constitutive activation of HERs and the downstream signaling molecules. This, in turn, leads to increased proliferation, alterations in the phosphorylation state of molecules implicated in the cytoskeleton, and modulation of neuregulin-responsive genes. All these events may drive the cell towards a more malignant phenotype.

Since far most studies related to the potential role of HRGs in regulating normal and oncogenic signals through its receptors have focused on its effects on mammary (cancer) cells, we restricted this review to the studies focusing on the involvement of HRG in normal mammary gland physiology and breast cancer. Its involvement in other (cancer) cell types is summarized in Table 1.

## Distinct roles for heregulin in mammary epithelial cells

### *Heregulin as a mammary gland mitogen regulating differentiation*

*In vivo*, in the mouse mammary gland, the only neuregulin-1 identified is heregulin-α. Its expression by the mammary mesenchyme, adjacent to lobuloalveolar structures, is suggestive for a role in the morphogenesis and ductal migration of mammary epithelial cells [65]. Expression is virtually absent in virgin glands, peaks at mid to late pregnancy and then sharply decreases after several days of lactation, becoming undetectable again during involution [65]. A crucial role for HRG in the differentiation of the mammary epithelium into secretory lobuloalveoli was confirmed by studies using mammary gland organ cultures [65] or mammary implants containing HRG [66]. This differentiation is characterized by an increase in nuclear size, large cytoplasmic vesicles, synthesis of milk protein (β-casein) and appearance of cytoplasmic fat droplets [65–67]. HRG-mediated upregulation and increased membrane targeting of Rab3A, a low molecular weight GTPase involved in vesicular trafficking, has been suggested to play a role in these processes [68]. Direct evidence for a role of HRG in lobuloalveolar development came from the generation of HRG-α knockout mice. In contrast to HRG-null mice, these mice survive to adulthood, but have transient defects in lobuloalveolar development, showing abrogated proliferation of luminal mammary epithelial cells during pregnancy and lactation. During lactation, this was accompanied by a dramatic reduction in β-casein expression [69]. Similar defects in lobuloalveolar development and lactation were observed in mice expressing dominant negative mutants of HER2 or HER4 in the mammary gland [70, 71].

In conclusion, HRG-α expression in the mammary gland may have a dual function, supporting survival and proliferation, while at the same time inducing differentiation. Deregulation of this balance between proliferation and differentiation, by alterations in expression levels of ligands or receptors, may contribute to transformation, tumor progression and metastasis. The dual action of HRG is also evident from its effects *in vitro* on cell lines: whereas sometimes proliferation is induced [10, 72–76], HRG treatment may lead cells to differentiation as well [8, 67, 73, 74, 77, 78]. Important determinants for these distinct effects likely include the system/cell line examined, methodology used, HRG isoform(s) involved, the concentration used, receptor expression profile, receptor–receptor interactions, as well as the intensity and duration of activation.

### *Heregulin as a growth-inhibiting, pro-apoptotic differentiation factor for mammary cancer cells*

Heregulin has been shown to exert differentiating effects on breast cancer cell lines in *in vitro* cell culture systems. As was observed in mammary glands, HRG may induce a differentiated phenotype of cultured cell lines, characterized by a flattened morphology, increased nuclear size, large cytoplasmic vesicles, synthesis of milk protein (β-casein), appearance of fat droplets and increased ICAM-1 expression [73, 79, 80]. In 3-D cultures inside collagen type I of immortalized non-tumorigenic mammary epithelial cells and SKBR-3 and T47D mammary carcinoma cell lines, HRG treatment induced a more differentiated phenotype [81]. When combined with retinoids, which are known inducers of differentiation in breast cancer cell lines [82], this differentiation was even more pronounced, possibly due to the induction of retinoid acid receptor-α by HRG in these cells [83]. The increase in differentiation was associated with elevated cell adhesion to cell surfaces, brought about by alterations in the level and distribution of integrins α2 and β1 [81]. Increased cell–cell adhesion, dependent on PI3K activity, has been observed in several breast cancer cells following exposure to HRG [84]. Since this increase in cell–cell adhesion was present in E-cadherin negative cell lines and could take place at 4 °C, it is cadherin-independent. Thus, also here, HRG-mediated modulation of integrins may play a role. In addition, using an MCF-7

*Table 1.* Involvement of HRG in (cancer) cells other than mammary (cancer) cells.

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|---|---|---|
| Activated monocytes | Activated monocytes may serve as a source of HRG. Since these cells cannot use the secreted HRG themselves, and since activation and infiltration of monocytes may be triggered by tumors, the secreted HRG may serve as a paracrine growth factor for the tumor cells. | 131 |
| Colon cancer | Autocrine HRG may give rise to constitutively activated HER2 and HER3, protecting against apoptosis and generating growth factor independence. HRG upregulates cyclooxygemase-2 (COX2) in colon cancer cells, resulting in increased prostaglandin E2 ($PGE_2$) secretion; and induces both uPA ($PGE_2$-dependent and -independent) and uPAR ($PGE_2$-independent). Both the growth-stimulatory and pro-invasive effects of HRG could be blocked by a specific COX-2 inhibitor, while also the uPA/uPAR system was necessary and sufficient for HRG-mediated invasion. | |
| | Using a model of FET differentiated colon carcinoma cells plated on Matrigel, HRG was found to affect colonic tissue remodeling, leading to the formation of three-dimensional, multiluminal structures. Formation of these lumen-containing structures was dependent on uPA/uPAR interaction as well and may also, at least in part, depend on upregulation of $PGE_2$. | 132–134 |
| Endometrial cancer | In normal endometrium higher HRG-$\alpha$ and $\beta$ levels are found in the secretory phase of the menstrual cycle as compared with the proliferative phase. Cytoplasmic HRG-$\alpha$ immunoreactivity was significantly higher in the stroma (which also sometimes displayed nuclear staining) than in the glands, whereas HRG-$\beta$ expression was weak to moderate in both the glands and stroma. In endometrial cance, HRG-$\beta$ levels remained unchanged, whereas decreased HRG-$\alpha$ levels paralleled loss of differentiation. | 135, 136 |
| Gastric cancer | HRG transcripts were detected in esophagus, stomach and duodenum, with no changes observed in gastric cancers. HRG-$\alpha$, which is secreted by gastric fibroblasts, was shown to be mitogenic for gastric epithelial cells, which may be of relevance since the high expression of HER3 and HER4 in gastric cancers. | 137, 138 |
| Glioma | HRG, expression is widely present in neurons and glia and has been found in gliomas. HRG treatment of glioma cells led to increased motility and invasion, which was accompanied by increased phosphorylation and association of focal adhesion kinase with HER2 | 139, 140 |
| Head and neck squamous carcinoma | 11 of 14 cells lines displayed HRG mRNA and secretion into the medium, resulting in an autocrine loop. The majority of these cell lines showed a mitogenic response to exogenously added HRG, which was accompanied by increased matrix metalloproteinase (MMP) production and invasion. | 141, 142 |
| Lung cancer | HRG may function as an autocrine and paracrine mitogenic factor for both normal lung epithelial cells and lung cancer cell lines. In normal human fetal lung, HRG expression is confined to the mesenchyme, suggesting a paracrine mechanism for stimulation of the adjacent epithelial cells. In contrast, in fetal lung explants, normal bronchial epithelial cell lines and lung cancer cell lines, HRG becomes expressed by the pulmonary epithelial cells as well, functioning as an autocrine, mitogenic ligand. | 143–149 |
| Malignant melanoma of soft parts | Increased expression has been observed in a cell line. | 150 |
| Melanoma | HRG may act as a potent paracrine growth factor for melanocytes. Multiple deregulations of the HRG/HER system were found in human melanoma cell lines. These included a lack of HRG responsiveness, owing to the absence of the HRG-receptor HER3, or owing to functionally inactive HER2, as well as autocrine growth stimulation by HRG. The latter resulted in constitutive activation of HER2 and HER3 and the downstream MAPK pathway. | 151 |
| Medulloblastoma | Nuclear HRG-$\beta$ staining has been observed in medulloblastomas, embryonic tumors of the cerebellum believed to arise from the cerebellar external granule cell layer. While newly acquired HRG-$\alpha$ expression was found in about one third of these tumors, almost all were positive for HRG-$\beta$, which is already present in normal human cerebellum during development. HRG was often co-expressed with HER4 and newly acquired HER2, suggesting the presence of an autocrine loop in the progression of medulloblastoma. | 37, 152 |
| Neuroepithelioma | An autocrine loop has been described in the SK-N-MC epithelioma cell line. | 153 |
| Ovarian cancer | HRG expression has been found in ovarian cancer cell lines, the majority of these showing a mitogenic response following treatment with HRG. Expression of HRG-$\alpha$1 and -$\beta$1, as measured by RT-PCR and immunohistochemistry, was present in about 80% of ovarian carcinomas, the levels being higher in serous carcinomas than in endometrioid carcinomas, indicating the potential of autocrine regulation. | 75, 76, 135, 154 |

*Table 1.* Continued.

| | | |
|---|---|---|
| Paget's disease | Normal keratinocyte-derived HRG-α has been suggested to play a crucial role in the invasion of large neoplastic cells originating in the mammary gland into the epidermis of the nipple, by inducing chemotaxis of the mammary cancer cells, which frequently overexpress HER2. | 155 |
| Papillary thyroid cancer | Immunohistochemistry revealed increased levels of HRG (α, β-1, β-2 and/or β-3) in both primary tumors and lymph node metastases, as compared to normal tissue. No association was found between HRG protein expression and clinical parameters in these patients. Apart from an increase in membrane and cytoplasmic HRG, the vast majority of tumors had positive nuclear immunostaining, which was never seen in normal thyroid tissue or in diffuse hyperplasia. This nuclear staining was only observed with an antibody recognizing the cytoplasmic tail of the HRG precursor, rendering papillary thyroid carcinoma a candidate system for the recently described nuclear signaling function of the HRG cytoplasmic tail. | 38 |
| Prostate cancer | Loss of HRG-α expression in prostatic adenocarcinoma has been observed in one study, whereas another study reported upregulation of the protein. HRG expression was absent in a panel of prostate carcinoma cell lines, but present in an immortalized non-transformed prostate epithelial cell line. Similar to the differentiation effects that were seen in some breast cancer cell lines, HRG reduced growth of the LNCaP prostatic cancer cell line, which coincided with increased contact-formation of these cells. | 78, 156, 157 |
| Skin squamous cell carcinoma | Conflicting reports have been published on the correlation between HRG expression and the differentiation state of keratinocytes and its mitogenic effects on these cells. The primary role of HRG production by keratinocytes does not seem to be growth stimulation, but would rather involve the stimulation of motility, playing a central role in wound healing processes. High expression, with constitutive receptor activation is seen in a skin squamous carcinoma cell line and has been suggested to play a role in hyperproliferative skin disorders as well. | 151, 158–165 |
| Transitional-cell carcinoma | Both normal human urothelium and a transitional-cell carcinoma show low HRG expression. In contrast to primary cultures of human urothelial cells, which were growth stimulated by a variety of EGF-like growth factors, but not by HRG, the latter was a potent mitogen for the transitional-cell-carcinoma cell line. | 166, 167 |
| Vestibular schwannoma | HRG, HER2 and HER3 are expressed, with presence of activated HER2, suggesting an autocrine loop. | 168 |

variant with a functionally deficient E-cadherin, we found that HRG could stimulate E-cadherin mediated adhesion as well. This was accompanied by decreased invasion of these cells into a precultured chick heart fragment (Stove et al., in press). On solid substratum, NRG-1 may induce a morphogenetic effect, rearranging epithelial islands into ring-shaped arrays with internal lumens [85], which may be a reflection of its function in lobuloalveolar morphogenesis *in vivo* [65]. Primary human mammary epithelial cells produce endogenous HRG [76], which possibly contributes to spontaneous differentiation of these cells.

HRG is involved in the development of the mammary gland, possibly through the regulation of the apoptotic process. In general, apoptosis may result from the net impact on pro- vs anti-apoptotic pathways. A critical determinant in this balance may be the expression levels of HER2, resulting in increased proliferation in cells that express low levels and leading to growth inhibition in HER2 overexpressing cells. This is evident from the fact that HRG is able to inhibit the growth of several breast and ovarian cancer cell lines that overexpress HER2 [8, 77, 80, 83, 86–90]. However, no growth inhibition of HER2-overexpressing cell lines was seen by others [76], indicating the important influence the test conditions may have on the outcome. HRG-mediated growth inhibition of the HER2 overexpressing SKBR3 breast cancer cell line relates to its induction of apoptosis, cell cycle G2-M arrest and cell differentiation [77, 86, 89]. These effects were suggested to be mediated via a mitochondrial pathway, involving downregulation of the anti-apoptotic protein Bcl-2 and activation of caspase-7 and -9, and being potentiated by inhibition of protein kinase Cα [90]. Alternative pathways by which HRG may induce apoptosis include the p70S6K/mTOR pathway, sustained activation of the JNK or MAPK pathway and late activation of p38 MAPK (day 2–3) [77, 87, 89] (Figure 2). Again, the pathway used may differ among different cell lines tested and even with a given cell line, may depend on the assay conditions. Although HRG-mediated induction of apoptosis and differentiation often coincide, different pathways may be involved, as exemplified by the involvement of the PI3K pathway in HRG-mediated induction of differentiation, but not apoptosis, of SKBR-3 cells [77].

In contrast to the above mentioned mechanisms, in which HER activation leads to growth inhibition and

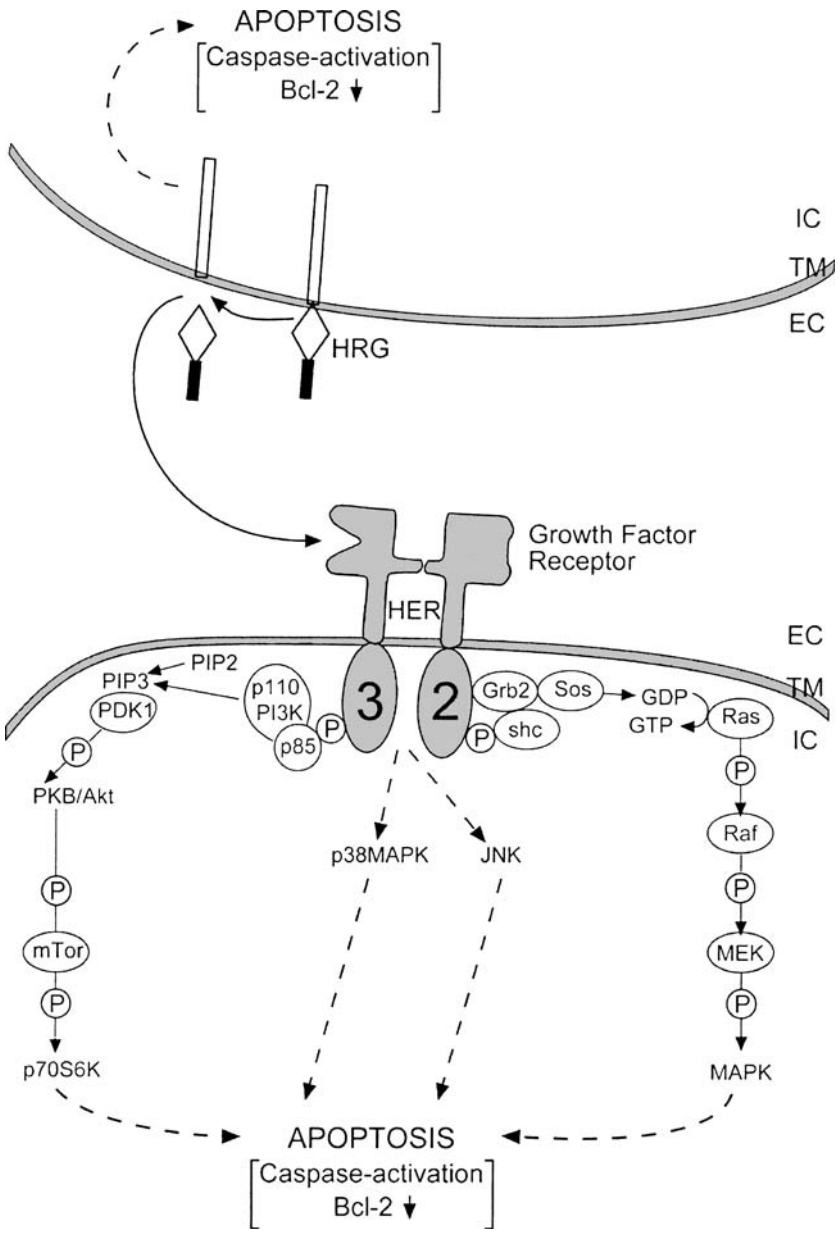


*Figure 2.* Schematic overview of the involvement of HRG in induction of apoptosis. Induction of apoptosis by binding of HRG to its cell surface receptors in a 'target cell' may involve a (combination of a) variety of signal transduction pathways (see text for details). Alternatively, back-signalling by the HRG intracellular tail may act as a dominant apoptosis-inducing signal in the 'donor cell' as well, the mechanism of which is incompletely understood.

apoptosis in selected cell lines, another mechanism for the pro-apoptotic activity of HRG has been suggested (Figure 2). This was based upon the identification of HRG-$\beta$-2b as a dominant apoptosis-inducing gene, the transmembrane domain and intracellular tail being important for this effect [88, 91, 92]. The fact that this HRG isoform could induce apoptosis in the absence of HER-binding, illustrates the dual function HRG might have: while cleavage may lead to secretion of HRG, which may have a transforming activity on HER-expressing cells (see below), the producing cell may undergo apoptosis when intracellular levels of the HRG cytoplasmic tail become too high. This selective killing of HRG-overexpressing cells might represent an auto-regulatory physiological mechanism protecting the organism against tumorigenesis. Consequently, loss of this sensitivity to apoptosis in HRG-overexpressing cells allows the continuous secretion of high amounts of HRG and may represent a step towards tumorigenesis. Consistent with these observations, epithelial cells from MMTV/heregulin-induced tumors exhibit high levels of apoptosis, in contrast to tumors induced in transgenic mice by HRG lacking the intracellular tail [88, 92, 93] Overexpression of HRG-$\beta$-2b in MCF-7 cells induced apoptosis as well, which depended on caspase activation and was accompanied by a downregulation of Bcl-2 [88]. In addition, constitutive overexpression of rHRG-$\beta$-2 in MCF-7 cells has been shown to markedly increase their sensitivity to doxorubicin and etoposide treatment [94].

Finally, it should be noted that inhibition of proliferation does not necessarily coincide with a less aggressive phenotype. This is evident from the effects of HRG and agonistic anti-HER2 antibodies on SKBR-3 mammary cancer cells: although proliferation of these cells is inhibited following receptor activation, invasion of these cells is increased [80].

### *Heregulin as a mitogenic, pro-invasive and metastatic factor in breast cancer*

As already mentioned above, the role of HRG in cancer has been particularly investigated in breast cancer. More than 60% of human breast cancers express estrogen receptor (ER) and hormone therapy is a commonly applied adjuvant therapy for breast cancer patients. Although ER expression is used to predict which patients will respond to hormone therapy, not all patients with positive ER will benefit from endocrine therapy [95, 96]. It is being increasingly recognized that altered expression of a variety of growth factors and their receptors may activate signaling pathways that influence ER signaling [96–98]. One of such factors is HRG. Conflicting reports on a possible role of HRG expression in human breast tumors have been published. Rajkumar and coworkers found in a histochemical study of locally advanced breast adenocarcinomas that absent or low levels of NRG-1$\alpha$ were associated with poorer prognosis as compared to tumors that had moderate to high levels of the protein [99]. In contrast, others have described elevated expression of HRG in a subset of breast cancer cell lines and in 25–30% of human primary breast cancers [100, 101], a percentage similar to that of HER2 overexpression in breast cancer [5, 6]. HRG expression in mammary tumors was found both in the stroma as in the tumor cells themselves, with stromal expression correlating with increased recurrence [102] or with activated protein kinase B (PKB/Akt), a downstream intermediate of PI3K, whose activation predicts a worse outcome among breast cancer patients [103]. Interestingly, HRG is particularly present in cell lines and tumor biopsies that do not overexpress HER2, inversely correlating with expression of estrogen receptor-$\alpha$ [101, 103]. The fact that the HRG-overexpressing population of breast tumors is distinct from that overexpressing HER2, combined with the ability of HRG to promote tumor formation in the absence of overexpression of HER2 (see below), suggests an important role for the HRG/HER system in the etiology of breast cancer. This may be of clinical significance, since nowadays only HER2 expression levels are being evaluated in tumor samples. Therefore, assessment of both receptor and ligand levels, or alternatively of receptor activation status [104], may provide a better basis for evaluation of the role these molecules play in particular cancers. This may result in a better identification of patients that may benefit from targeted therapies.

HRG is a potent mitogen for most breast cancer cell lines, provided they express HERs. Using a panel of different assays applied on a series of breast cancer cell lines with varying HER2 levels, Lewis et al. [75] and Aguilar et al. [76] demonstrated a clear growth-stimulatory role for HRG, both *in vitro* and *in vivo*. Supporting these findings, the mitogenic action of exogenous HRG was less pronounced or absent in cells already producing HRG in an autocrine way, although increasing HER2 levels in these cells increased their responsiveness. Its potency is evident from its ability to act as a dual specificity growth factor for the human mammary epithelial cell line MCF-10A, in which it drives signal transduction pathways that normally require both EGF and insulin-like growth factor-I [105]. The mitogenic action of HRG is likely to be achieved through the combined action of multiple pathways, including PI3K, MAPK and p38MAPK pathways [106, 107]. These affect proteins involved in cell cycle progression, as exemplified by the promotion of expression of c-Myc, A-, E- and D-cyclins and $p21^{cip1}$, activation of cyclin-dependent kinases and phosphorylation of the retinoblastoma protein (pRB) [106, 108]

Multiple studies suggest that HRG induces breast cancer progression towards an aggressive phenotype, as determined by hormone independence, antiestrogen resistance (loss of ER function and response), tumorigenicity [109], invasion [110–112], and metastasis [113–115]. It does so directly by increasing cell motility or indirectly, via the regulation of genes that regulate and control malignant progression (Table 2; see below).

In breast cancer cells, HRG can block both estradiol (E2) action and ER function. HRG antagonizes the E2-mediated downregulation of HER2 and is capable of enhancing tamoxifen-induced stimulation of the receptor [109, 116, 117]. HRG supplementation of ER-positive breast cancer cell lines results in resistance to the growth inhibitory effects of antiestrogens, which is more pronounced with partial than with full antiestrogens [118]. In addition, treatment/transfection of the ER-positive, E2-dependent MCF-7 breast cancer cell line with HRG-$\beta$-1/2 resulted in a loss of E2 dependence and acquisition of antiestrogen resistance both *in vitro* and *in vivo* [109, 117, 114, 119]. Inoculation of HRG-supplemented or HRG-producing MCF-7 cells in the mammary glands of ovariectomized nude mice resulted in spontaneous, E2-independent tumor formation [76, 114]. These models mimic in fact what is seen in many breast cancer patients: ER-positive tumors acquire antiestrogen resistance during the course of the treatment, while still retaining some levels of ER. The fact that other growth factors, such as transforming growth factor-$\alpha$, which are also mitogenic for MCF-7 cells, cannot support this E2-independent growth, pleads for the strength of HRG as a transforming factor [120].

*Table 2.* Genes, whose functional relevance has been described, that are regulated by HRG.

| Function | Gene | Cell-type | Level | Pathway | Reference |
|---|---|---|---|---|---|
| Transcription | ATF4 | BC, CC | TC | ERK | [180] |
| | GADD153 | BC | TC protein ($\uparrow t^{1/2}$) | | [181] |
| | HIF-1α | BC | TL | PI3K (AKT/FRAP) | [170] |
| | hnRNP K | BC | mRNA | | [179] |
| | MTA1 | BC | TC | | [115] |
| | RARα | BC | mRNA | | [76] |
| Translation | EF-1α | BC | TC | p38MAPK ERK | [182] |
| RNase and helicase activities | G3BP | BC | TC | | [186] |
| Chaperone | HSP70 | BC | mRNA | | [183] |
| | Calnexin | BC | TC | | [184] |
| (Lymph)angiogenesis | VEGF-A | BC, HNSCC | TC post-TC | PI3K (Pak1)(HIF-1α) ERK P38MAPK | [166–171, 173] |
| | VEGF-C | BC, HNSCC | TC | P38MAPK (NFκB) | [172, 173] |
| Migration | Cyr61 | BC | mRNA | | [102] |
| Adhesion | ICAM-1(CD54) | BC | mRNA | PKC | [72, 73] |
| | HCAM (CD44) | BC | | | [73] |
| | Integrin alpha 5/6 | KC | | | [146] |
| | Integrin α6β4 | KC | | | [147] |
| Migration | AMF | BC, CC | TC | P38MAPK ERK | [177] |
| Vesicular Trafficking | Rab3A | BC | TC | PI3K | [61] |
| Prostaglandin synthesis | COX-2 | CC (not BC,OC) | TC | | [136] |
| Proteolysis | MMP-9 | BC, HNSCC | TC | PKC p38MAPK (ERK) | [104, 106, 107, 155] |
| | MMP-1, -3, -7, -10, -11, -13 | HNSCC | TC post-TC | | [155] |
| | uPA | BC, CC | TC | ERK NFκB | [103, 138] |
| | uPAR | BC, CC | TC | ERK p38MAPK | [103, 138] |
| Differentiation marker | β-casein | BC | mRNA | | [58, 59, 60, 62] |
| Cell cycle | c-Myc | BC, OC, CC | TC | | [98, 100, 136] |
| | p21cip1 | BC | TC | PI3K p38MAPK | [98, 100] |
| | Cyclin A | BC | TC | | [82, 100] |
| | Cyclin B | | | | [82] |
| | Cyclin E | BC | TC | | [100] |
| | Cyclin D1 | BC | TC | PI3K ERK p38MAPK | [98, 100] |
| | Cyclin D2 | BC | mRNA | PI3K ERK p38MAPK | [98] |
| | Cyclin D3 | BC | mRNA | PI3K ERK | [98] |
| Downregulated genes: | | | | | |
| Transcription | ERα | BC | TC | PI3K/AKT ERK | [109, 113, 114] |
| Ubiquitin polymerization | BRCA1 | BC | mRNA protein | PI3K | |
| Cytoskeleton | Keratin 1, 10 | KC | mRNA | | [147] |
| Differentiation marker | Involucrin, loricrin | KC | mRNA | | [147] |

Upregulated genes are indicated in the upper part of the table, downregulated genes are at the end of the table. Abbreviations used for cell types: BC, breast cancer; CC, colon cancer; HNSCC, head and neck squamous cell carcinoma; OC, ovarian cancer; KC, keratinocytes; for level of regulation: mRNA: altered mRNA levels, but mechanism not specified, TC: altered transcription; TL: altered translation, $\uparrow t^{1/2}$: increased halflife. When regulatory level or pathway is not indicated, it was not described in literature.

Depending on the conditions applied, both suppression [109, 116, 121] and activation [117, 122] of the ER signaling pathway have been described. Both scenarios, likely resulting in differences in activation of several pathways, may lead to E2-independency (Figure 3).

In the first scenario, low concentrations of HRG decrease ERα levels, activity and binding to its response element in a DNA mobility shift assay, as well as estradiol-induced growth of breast cancer cell lines [109, 116, 121]. A molecular mechanism,

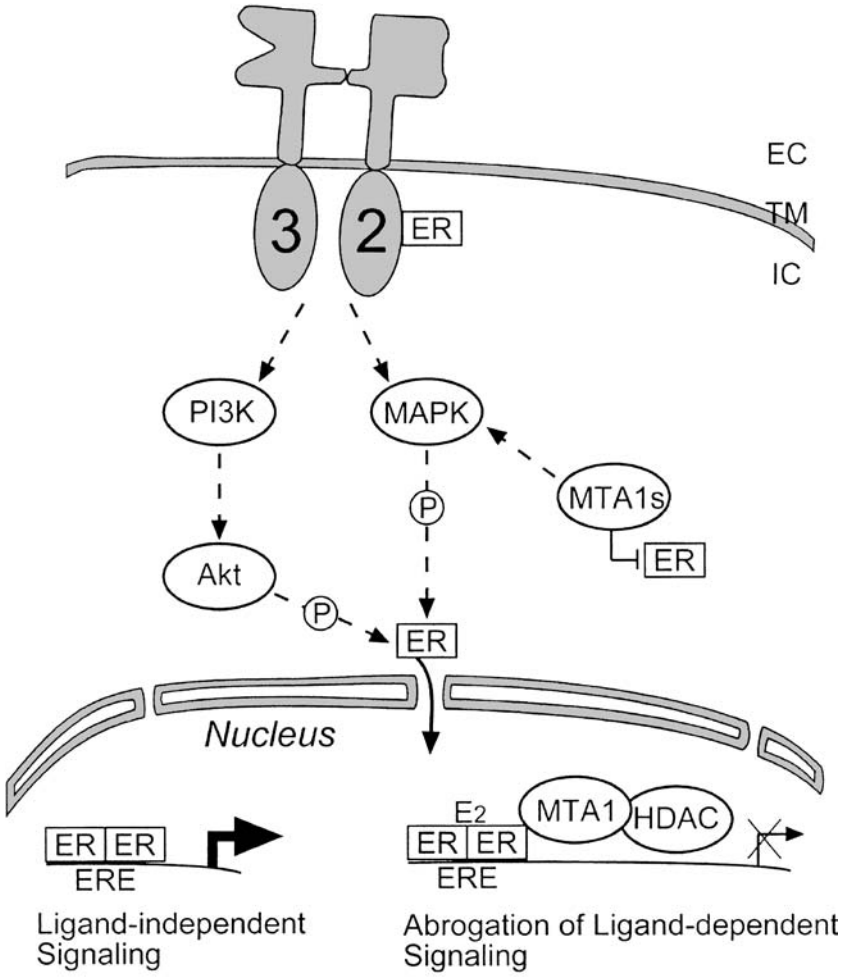


*Figure 3.* Schematic overview of the possible effects of HRG signalling on ER signalling. Binding of HRG to its receptors leads to activation of a variety of signal transduction pathways, which may be dependent on the association of ER with these receptors. This may eventually lead to phosphorylation of ER, resulting in ligand-independent signalling. MTA1, a target gene of HRG signalling, may suppress transcription of ER target genes by recruiting HDACs to ERE in their promoters, while its splice variant MTA1s may sequester ER in the cytoplasm.

involving upregulation of metastasis-associated protein 1 (MTA1), has been proposed for the HRG-mediated suppression of E2 response elements (ERE) and disruption of E2 responsiveness [123]. In accordance with the correlation between MTA1 expression and the metastatic potential of several human cell lines and tissues [124] these authors found that MTA1 was increased in Harderian-gland tumors in MMTV-driven HRG transgenic mice and could induce invasion *in vitro* [123]. By physically interacting with the activation factor domain of ERα and with histone-deacetylase (HDAC) 1 and 2, MTA1 recruits HDAC activity to ERE's, suppressing transcription of target genes containing these ERE's. Alternatively, MTA1s, an MTA1 splice variant containing a unique RLILL motif, has been shown to prevent E2-induced nuclear translocation by sequestering ERα in the cytoplasm. Expression of this splice variant was higher in breast cancers and correlated with absence of nuclear ER [125]. Hyperactivation of MAPK, accompanied by loss of ERα without activation of ERα responsive genes, may be a cause of estrogen unresponsiveness as well [126]. Interestingly, cells expressing the MTA1 splice variant had increased levels of MAPK activation, suggesting that expression of this protein may confer unresponsiveness to E2 while stimulating other pathways at the same time [125].

For the second scenario leading to E2-independency, in which ligand-independent ER activation occurs, Stoica et al. [122] recently presented an interesting mechanism. Using MCF-7 cells, they showed that both the binding of HRG to HER3/HER2 and the presence and activity of membrane ERα (which may be physically associated with HER2 [127]) are required for PI3K/Akt activation. Akt activation, on its turn, leads to phosphorylation of ERα and a decrease of ERα mRNA and protein, coinciding with increased expression of progesterone receptor (PR) and pS2, two markers of ERα signaling [117, 122]. Interestingly, further demonstrating the intense cross-talk between HERs and ERα, they also showed that ERα-mediated PI3K/Akt activation and activation of PR and pS2 target genes was abrogated by blocking HER2. Also the MAPK pathway is thought to play

an important role in mediating the effect of HRG on cell proliferation and ERα. A possible crosstalk between the ER and MAPK pathways is supported by studies in which ERα was found to be phosphorylated by MAPK and in which inhibition of the MAPK pathway restored the sensitivity to antiestrogens [128, 129]. In conclusion, HRG may provide cells with a phenotype that depicts the passage from an initial E2-responsive and antiestrogen-sensitive tumor cell to a later step in carcinogenesis, resembling the common progress of many human breast tumors.

HRG promotes tumorigenicity and metastasis of breast cancer cells that do not overexpress any of the HERs [109, 114]. An increase in the invasive phenotype of MCF-7 cells exposed to HRG was found both *in vitro* and *in vivo*. In contrast to normal MCF-7 cells, HRG-expressing MCF-7 cells injected into the mammary fat pad of nude mice metastasize to the axillary lymph nodes and induce preneoplastic transformation of the adjacent mouse mammary epithelium [114]. Further substantiation for a role of HRG as a transforming and tumor progression factor was provided by transgenic mouse models in which HRG-β-2c, either full length or lacking most of the cytoplasmic tail, was expressed under the control of the murine mammary tumor virus (MMTV) promoter. Overexpression of HRG in the mammary glands of these mice induces a persistence of the terminal end buds in the transgenic virgin females. More importantly, multiparous female mice have extensive mammary hyperplasia and develop mammary adenocarcinomas at 12–15 months of age [94, 130]. In addition, less apoptosis was observed in tumors from transgenic mice that overexpress the extracellular region of HRG, as compared with tumors from mice overexpressing full-length HRG, in line with a proposed role for the HRG cytoplasmic tail in apoptosis [94]. Conversely, upon treatment of HRG-overexpressing breast cancer cell lines with HRG blocking antibodies, *in vitro* growth, motility and invasion of these cells is decreased [119]. This was further confirmed by Tsai et al. [115], using stable HRG-antisense transfectants of MDA-MB-231 mammary carcinoma cells, endogenously expressing HRG. In these cells, reduction of HRG expression abrogated the pre-existing autocrine loop, resulting in decreases in proliferation, anchorage-independent growth, motility and invasion *in vitro*, the extent of these mirroring the decrease in HRG expression. Moreover, inoculation of these cells into the mammary fat pads of athymic nude mice resulted in tumors that were significantly smaller than those induced by the parental cells and that did not metastasize [115].

## Involvement of neuregulin-responsive genes in malignant progression

Several neuregulin-responsive target genes are known candidate genes for malignant transformation. These genes may fulfill important roles in distinct steps of cancer progression. One of the important steps for tumor progression, invasion and eventually metastasis is the destruction of the extracellular matrix that separates the epithelial and stromal compartments. Several proteases have been involved in this process. Of these, the matrix metalloproteinases (MMPs), a family of more than 20 zinc-dependent endopeptidases, are involved in the breakdown of extracellular matrix during tissue remodeling. Aberrant expression of MMPs has been widely recognized in cancer progression, being involved in tumor cell invasion and metastasis [169]. One such enzyme is MMP-9 (gelatinase B), which plays a role in the degradation of type IV collagen (gelatin) and is highly expressed in breast carcinomas, correlating with increased metastatic potential [170, 171]. HRG is known to upregulate MMP-9 expression and enzymatic activity [80, 112, 114, 115, 142]. Multiple signaling pathways, including PKC, p38MAPK and ERK1/2 pathways, were found to contribute to this upregulation [112]. Moreover, using a specific MMP-9 inhibitor, the invasive phenotype of HRG-expressing cells can be blocked [114], thus providing evidence for an important role for MMP-9 in HRG-induced invasion. Other MMP's that were upregulated following treatment of head and neck squamous carcinoma cell lines with HRG included collagenases, stromelysins and matrilysins, but not gelatinase A (MMP-2) [142]. In both breast and colon cancer cell lines, HRG upregulates the serine protease urokinase plasminogen activator (uPA) and its receptor (uPAR) [111, 134]. Following HRG treatment, an increase in membrane-bound uPA was observed, being redistributed to focal adhesion points, most frequently at the leading edges of the cell. A role for the uPA/uPAR system in HRG-mediated invasion could be deduced from the fact that invasion could be counteracted by blocking uPAR [134]. These findings are consistent with the frequent expression of uPA/uPAR at the leading edge of tumor cells and its correlation with tumor cell invasiveness and aggressiveness [reviewed in 172]. Whether uPA plasminogenic activity or uPAR-induced signaling is involved in the effects of HRG, has not been studied. It should be noted that the induction of proteolytic enzymes by HRG is not observed in all cell lines under all conditions tested [173]. For instance, in the invasive LM3 mouse mammary adenocarcinoma cell line, HRG treatment resulted even in a decreased activity of uPA and MMP-9, correlating with decreased proliferation and migration [174].

Increased *de novo* formation of vascular and lymphatic systems contributes to several aspects of tumor progression: they do not only supply the tumor with fresh blood, which is a key factor for the growth and survival of solid tumors, they comprise systems by which tumor cells can easily spread to nearby or distant tissues as well. Critical growth factors known to mediate these processes belong to the vascular

endothelial growth factor (VEGF) family. Overexpression of VEGF family members has been detected in tumor specimens and correlated with an advanced invasive phenotype [175]. HRG has been implicated in (lymph)angiogenesis by its mitogenic effects on endothelial cells and its induction of *VEGF-A and -C* in breast cancer cells *in vitro* and *in vivo* [176–182]. This induction was not observed in normal mammary or bronchial epithelial cells [178]. HRG treatment of head and neck squamous carcinoma cells resulted in an upregulation of VEGF-A, and -C, did not affect VEGF-B levels, and downregulated VEGF-D [183]. Interesting in this respect is that such differential regulation of different VEGF family members has been associated with malignant progression and lymph node metastasis in lung cancers [184]. Several mechanisms have been proposed for the HRG-mediated increase in VEGF-A and -C. For VEGF-A, upregulation may occur in a PI3K-dependent manner, by activation of p21-activated kinase 1 (Pak1); by increased translation of hypoxia inducible factor (HIF)-1$\alpha$, which, together with HIF-1$\beta$, acts as a transcriptional activator of the VEGF-A gene; and/or by MAPK-dependent activation of Sp-1 and AP-2 transcription binding sites in the VEGF-A promoter [177, 180, 181]. Consistent with this, it has been shown that the Sp-1 transcription factor is stimulated by phosphorylation following HRG-induced HER activation [185]. Alternatively, or in combination, HRG-induced transient activation of p38MAPK results in enhanced VEGF-A and -C transcription and secretion of VEGF protein in breast cancer cells [179, 182]. For the lymphangiogenic VEGF-C, this was shown to depend on a p38MAPK-dependent phosphorylation and inactivation of I$\kappa$B$\alpha$, releasing the transcription factor NF$\kappa$B, which, after nuclear translocation, binds to and activates an NF$\kappa$B-binding site in the VEGF-C promoter [182].

Another angiogenic factor that is upregulated by HRG is Cyr61, a cysteine-rich ligand that associates with the cell surface and extracellular matrix and functions as a ligand for $\alpha v\beta 3$ integrin, promoting cell adhesion and migration. Cyr61 was overexpressed in about 30% of breast tumor specimens, its expression in breast cancer cell lines correlating with the presence and absence of HRG and ER, respectively [110]. Moreover, Cyr61 overexpression was shown to support estrogen-independent growth *in vitro* and *in vivo* [186]. Apart from these angiogenic molecules, angiogenic effects have been attributed to MMP-9 and the uPA/uPAR system as well, extending the roles these molecules may play in HRG-induced tumorigenesis. Tumor cells must adhere to endothelial cells and interact with components of the extracellular matrix to establish metastatic foci. In this context, the upregulation of cell adhesion molecules may contribute to increased invasion by HRG. Examples include *ICAM-1 (CD54)* and *HCAM (CD44)*, which are both upregulated following HRG treatment of SKBR-3 breast cancer cells [79, 80]. HRG increased adhesion to plastic and induced invasion of SKBR-3 cells. This invasion could be partially blocked by either anti-CD44 or anti-CD54 antibodies, indicating a role for these molecules in the invasion process [80].

HRG treatment of breast cancer cells results in the upregulation of autocrine motility factor (AMF), a growth factor whose expression has been proposed to play a role in metastasis and correlates with disease progression in various cancers [187, 188]. Roles for HRG-induced AMF include the promotion of motility, since HRG-induced scattering and invasion could be reduced upon blocking AMF function, as well as growth support, based on the additive growth inhibitory effect of a HER2-blocking antibody and AMF inhibitors.

Amplification of the HRG signal may occur by upregulation of proteins involved in transcription, since these, on their turn, may activate a subset of target genes. Among the proteins involved in transcription that have been shown to be upregulated by HRG are HIF-1$\alpha$ [180], heterogeneous nuclear ribonucleoprotein K (hnRNP K, a known activator of c-myc) [189] and the basic leucine zipper transcription factors activating transcription factor 4 (ATF4) [190] and GADD153 (growth-arrest and DNA-damage 153) [191]. Interestingly, the latter could activate transcription of the $\beta$-casein promoter in a Stat5a-dependent way. Since GADD153 expression in the mouse mammary gland is predominantly restricted to early lactation, it represents a good candidate gene for the induction of $\beta$-casein by HRG *in vivo*. Also translation may be enhanced, since elongation factor-1$\alpha$ (EF-1$\alpha$), a ubiquitously expressed protein that is involved in the elongation cycle during translation, was identified as a HRG target gene as well. Upregulation of this protein was mediated by a Sp-1 transcription site in its promoter and involved increased chromatin acetylation [192]. Further upstream, upregulation of proteins with chaperoning function may facilitate correct protein folding and processing. Examples include the induction of *heat shock protein-70* and *calnexin* [193, 194].

Stimulation of cells with HRG does not lead exclusively to upregulation of responsive genes. As already mentioned above, estrogen and progesterone receptor levels decrease following HRG treatment. *BRCA1*, a tumor suppressor gene whose inactivation is associated with a high incidence of familial breast and ovarian cancer, represents another HRG-repressed gene [195]. HRG treatment of breast cancer cells was shown to affect BRCA1 in two ways: first, receptor activation results in a phosphorylation of BRCA1 in a PI3K-dependent manner and by cyclin-dependent kinase 4; second, HRG stimulation causes a decrease in BRCA1 mRNA levels, which is dependent on protein synthesis. Both effects were enhanced upon culturing the cells on extracellular matrix.

**Non-genomic effects of HRG on cell motility**

Members of the Rho family of small GTPases have emerged as key regulators of the actin cytoskeleton, which has been implicated in many cellular functions, including motility, chemotaxis, cell proliferation, differentiation, endocytosis, secretion and cell polarization [197]. HRG stimulation of non-invasive breast cancer cells enhanced the conversion of globular actin to filamentous actin and the formation of membrane ruffles, stress fibers, filopodia, and lamellipodia, which was accompanied by increased cell migration [198]. These prominent cytoskeletal changes induced by HRG are accompanied by dramatic changes in phosphorylation status of several proteins present in focal adhesions, regions where cells make integrin-mediated contacts with the extracellular matrix and that serve as anchorage points for actin stress fibers. Concomitantly, $\beta$-1-integrin-mediated adhesion to extracellular matrices is rapidly upregulated [199]. A protein which is localized at focal contacts is *paxillin*, a cytoskeletal phosphoprotein that becomes phosphorylated on a serine residue following HRG treatment. This occurs in a p38MAPK-dependent manner and results in disassembly of paxillin from the focal adhesion complexes during HRG-induced cell shape alterations and motility [200]. In addition, HRG triggered a redistribution of paxillin to the perinuclear regions. Regulation of cytoskeletal reorganization and cell migration by HRG were found to be mediated by a PI3K-dependent activation of p21-activated kinase-1 (Pak1), which was redistributed to the leading edges of motile cells upon HRG treatment. Another target of HRG signaling includes *Tiam1*, a guanine nucleotide exchange factor that has been implicated in regulation of cell migration [201]. HRG stimulation of breast cancer cells induces phosphorylation and redistribution of Tiam1 to membrane ruffles. This is accompanied by loosening of intercellular junctions and tyrosine phosphorylation of $\beta$-catenin, which is redistributed from the membrane to the cytosol and nucleus, where it may serve as a transcription factor. Also *focal adhesion kinase* (FAK), a well-characterized protein in focal adhesion complexes that has been implicated in the regulation of cell motility, invasion, adhesion and anti-apoptotic signaling [202], is subject to regulation by HRG [203]. At low concentrations, in which increased cell adhesion and formation of well-defined focal points takes place, phosphorylation of FAK was induced. At these concentrations, association of FAK with HER2 could be observed. High doses of HRG, which led to increased migratory potential of the cancer cells, resulted in a dephosphorylation of FAK. This was accompanied by a decreased association of FAK with HER2, but an increased association of the tyrosine phosphatase SHP-2 with the activated receptor, which may be responsible for the observed dephosphorylation of FAK. HRG treatment of breast cancer cells resulted in phosphorylation of RAFTK (related adhesion focal tyrosine kinase), a cytoplasmic protein related to focal adhesion kinase, leading to its localization in a multiprotein complex containing HER2, Src, and the GTPase activating proteins (GAPs) p190 RhoGAP and RasGAP. Formation of this complex, in which p190RhoGAP becomes phosphorylated by Src, has been shown to play a role in various aspects of HRG function, including HRG-induced invasion and MAPK activation [204]. Alternatively, HRG treatment increased the association of RAFTK with Csk homologous kinase (CHK), which, via inhibition of RAFTK phosphorylation, may negatively regulate HRG-mediated signaling [205]. Consistent with this, CHK was shown to inhibit *in vitro* cell growth, transformation and invasion induced upon HRG stimulation [206].

**Regulation of HRG expression**

Relatively little is known about the regulation of HRG expression. This may be due to the complexity of the NRG-1 gene, where the expression of the distinct isoforms is most likely regulated through the usage of alternative promoters. However, upregulation of NRG expression has been described in several cell systems. As can be expected from its prominent increase in the mammary gland during pregnancy [65], HRG expression is under the influence of hormonal control. Thus, HRG not only influences steroid receptor signaling, it is itself subject to regulation by *steroid receptors*. Upregulation of HRG expression in epithelial tumor cells was observed in an *in vivo* model of mammary carcinogenesis induced by the synthetic progestin medroxyprogesterone acetate (MPA) [207]. This upregulation was found in MPA-dependent and -independent ER/PR-positive ductal tumors but was absent in ER/PR-negative lobular carcinomas. These findings are consistent with the suggestion of Jones et al. [66] that progesterone might contribute to the HRG-induced lobuloalveolar development *in vivo*. In the mouse mammary gland, stromal HRG expression required the presence of adjacent epithelia, suggesting a reciprocal interaction between these cell types, possibly mediated by secreted proteins [65]. Growth factors such as *keratinocyte growth factor* (KGF) and *hepatocyte growth factor* (HGF) and *EGF*, but not insulin-like growth factor-1 or transforming growth factor-$\beta$ were able to upregulate HRG expression in keratinocytes, although the underlying mechanisms are not clear. This induction was confirmed in two *in vivo* models of tissue repair in mice: in full-thickness skin wounds, following locally increased KGF production, and in kidney after partial hepatectomy, following elevation of increasing HGF levels [164]. *Cross-induction*, i.e. upregulation of an EGF-like growth factor following stimulation with a family member, frequently occurs in human carcinomas. As a result, two or more EGF-like peptides are often co-expressed, leading to a sustained mutual co-amplification mechanism. Using a

squamous carcinoma cell line as a model, O-charoenrat et al. found that HRG mRNA was strongly upregulated following treatment with any of the EGF-like ligands tested (EGF, transforming growth factor-α, betacellulin, heparin-binding-EGF, amphiregulin and HRG-β-1 itself). This was suggested to involve both transcriptional and post-transcriptional mechanisms [141]. Consistent with its identification as a factor released by *ras*-transformed fibroblasts [8, 9], the insertion of *activated H-* or *K-ras* also resulted in upregulation of NRG-expression in breast cancer cells [208]. However, our analysis in melanoma cells, in which activated *N-ras* was present, could not provide a role for Ras-signaling in upregulation of HRG expression (unpublished data). Another oncogene, *cph*, was suggested to underlie the high HRG expression observed in a hamster embryo fibroblast cell line [209]. Regulation of HRG expression by its co-receptor *HER2* has been described. However, whereas in ovarian cancer cell lines HER2 overexpression induced HRG, its expression in both normal immortalized and cancer-derived breast cell lines significantly decreased or remained unchanged [76]. Stimulation of a monocytic cell line and normal peripheral blood monocytes with *phorbolester* resulted in increased HRG mRNA and protein levels [131]. Consistent with this, we found increased levels of HRG mRNA in primary human melanocytes cultured in the presence of phorbolester (unpublished data). In keratinocytes, a decrease in the level of HRG transcripts occurred upon attaining culture confluence, conversely correlating with the levels of HER2 and HER3 in these cells [159].

In addition to the several pathways that have been described to upregulate HRG expression, improvement of its release by the cells may contribute to its effects as well. This was evident from a study in which growth inhibition of squamous cell carcinoma cell lines by MMP inhibitors could be attributed to their inhibitory effects on the release of EGF-like ligands, including HRG [210]. Since the HRG precursor could not be cleaved anymore, no HRG was available anymore to function in an autocrine loop. Also in a physiological context, this metalloproteinase-mediated cleavage of neuregulins is important, as exemplified by Meltrin-β (ADAM19) knockout mice, in which heart development is compromised because of a lack of neuregulin-mediated paracrine signaling [211].

## The *NRG1* locus as a target of recurrent chromosome translocation breakpoints

Recently, disruption of the 1.1 Mb-spanning *NRG1* locus at 8p12 was found in several human breast (5 out of 34) and pancreas cancer cell lines (2 out of 9) [212]. These breakpoints differed among the different cell lines. In the human breast cancer cell line MDA-MB-175, a t(8;11) translocation leads to a fusion product consisting of the N-terminal part of DOC4, a stress-induced gene of unknown function located on chromosome 11q13, and HRG [212–214]. Expression of this novel secreted chimeric ligand, coined γ-heregulin, is under the control of the DOC4 promoter, thus generating an autocrine loop which gives rise to constitutive activation of HERs in these cells [215]. A survey for this particular translocation in a series of 141 breast cancer patients did not reveal any abnormalities [216]. However, since in all of the cell lines with disruption of the *NRG1* locus the breakpoint differed, thorough examination of the entire *NRG1* locus rather than a screening for a specific breakpoint is needed. Although the MDA-MB-175 example likens NRG1 to be an oncogene, in the majority of the cell lines with a *NRG1* locus breakage the result of the translocation is unknown. Although no correlation could be found between the presence of a breakpoint and the (pattern of) secretion of NRG isoforms, it is interesting to mention that in the initial characterization of one of the affected cell lines, SUM-52PE, a juxtacrine HRG autocrine loop was described [217]. Yet, it remains to be determined whether in this and other cell lines the chromosomal translocation directly contributes to malignancy. If so, either activation by promoter swapping or protein fusion, or inactivation may contribute to tumor progression. Alternatively, it is possible that, depending on the site of breakage and the isoform(s) targeted, cell behavior is affected in a different way. The observation that loss of heterozygosity at microsatellite markers from region p11-21 of chromosome 8, which encompasses the *NRG1* locus, is a frequent event in microdissected breast tumor samples, but not in peritumoral cells [218], combined with the recent identification of breakpoints in the HRG locus in samples from breast cancer patients [219], offers an intriguing possibility that disruption of this locus may indeed represent an important event in cancer. In conclusion, the evidence that the *NRG1* locus may encompass a fragile site in cancer warrants further research, both in the clinic, verifying whether rearrangement of this locus is a recurrent theme in biopsies from different cancer patients, as in basic research, elucidating the mechanisms underlying the breakage and resulting from it.

## Is there a role for neuregulins-2, -3 and -4 in cancer?

As is evident from the above, the vast majority of publications describing the role of NRGs in cancer focuses on members of the NRG-1 family. Recently, however, some attention is being given towards the other neuregulins and their possible role in malignant progression. Like neuregulin-1, also neuregulin-2, -3 and -4 were described to stimulate cell proliferation, provided the cells express the appropriate receptors [21, 23, 220, 221]. Moreover, suggesting a similar dual function as has been described for neuregulin-1, also neuregulin-2 can induce differentiation of breast cancer cells *in vitro*,

the strength and biochemical characteristics of the effect depending on the isoform applied [222–224]. The only report on expression of neuregulins-2, -3 or -4 in cancer is an immunohistochemical study of breast cancers using home-made polyclonal antibodies to each of the neuregulin family members [225]. In this study, a tendency of co-expression of multiple neuregulins in one tumor was observed. However, no correlation with clinical parameters was apparent. Recently, an anti-angiogenic role has been attributed to a neuregulin-2/NTAK isoform, NTAK$\gamma$, on basis of its ability to inhibit proliferation of endothelial cells [226]. Since this effect was not mediated by its EGF-like domain but, instead, depended on its N-terminal domain, this may open a wide array of novel functions attributed to domains outside the EGF-like domain.

## Conclusion

In conclusion, the neuregulin-HER system is of critical importance in regulating a variety of physiological events. A tightly regulated balance exists between differentiating and growth-promoting effects of neuregulins. Deregulation of this balance may contribute to malignant transformation. While most studies have focused on overexpression of HERs as a mechanism leading to constitutive receptor activation, it is becoming increasingly clear that, in the presence of normal receptor levels, aberrant constitutive signaling may occur as well, due to the presence of autocrine or paracrine loops. While the action of neuregulins *in vivo* is typically paracrine, being expressed in mesenchymal tissues adjacent to epithelia, epithelial tumors frequently show gain of expression, increased (sensitivity to) paracrine signaling and/or disruption of spatial control, all of which may contribute to the transformation process. This transformation is likely mediated by activation of a wide array of signaling events, leading to changes in gene expression, as well as rapid alterations in the actin cytoskeleton. Although relatively little is known about the mechanisms leading to increased HRG expression, the recent identification of the *NRG1* locus as a target of recurrent chromosome breakpoints in cancer cell lines and in patient samples offers an intriguing possible explanation. The characterization of HRG as a constitutive activator of HERs in a variety of tumors may offer opportunities for targeted therapies in these cancers. Therapeutic approaches targeting ligand-overactivated receptors may include the use of monoclonal antibodies interfering with receptor heterodimerization [227, 228], as well as the use of selective small molecule tyrosine kinase inhibitors [reviewed by 229–231]. Although *in vitro* studies are promising, for most of these the success in the clinic for specifically targeting ligand-overactivated receptors in a variety of tumors remains to be established.

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