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Review

Role of heregulin in human cancer

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Abstract. Heregulin (HRG) is a soluble secreted growth factor, which, upon binding and activation of ErbB3 and ErbB4 transmembrane receptor tyrosine kinases, is involved in cell proliferation, invasion, survival and differentiation of normal and malignant tissues. The HRG gene family consists of four members: *HRG-1*, *HRG-2*, *HRG-3* and *HRG-4*, of which a multitude of different isoforms are synthesized by alternative exon splicing, showing various tissue distribution and biological activities. Disruption of the physiological balance between HRG ligands and their ErbB receptors is implicated in the formation of a variety of human cancers. The general mechanisms involved in HRG-induced tumorigenesis is discussed.

Keywords. Heregulin, growth factor, signaling strategies, ErbB, tumorigenesis.

Introduction

Heregulin [HRG, also called Neuregulin (NRG), Neu differentiation factor (NDF), glial growth factor (GGF), and acetylcholine receptor-inducing activity (ARIA)] was first cloned and characterized in rat [1, 2] and in human [3] as a putative ligand for the ErbB2 transmembrane receptor tyrosine kinase. However, it was shown later that ErbB2 functions as a co-receptor, forming a heterodimer with the actual HRG receptors, ErbB3 and ErbB4, thereby being activated indirectly upon ligand binding [4–6]. The HRG gene family consists of four members, *HRG-1*, *HRG-2*, *HRG-3* and *HRG-4* [3, 7–10], encoding at least 26 different HRG isoforms through alternative splicing in different species [11]. These different types of HRG and its isoforms, in general, have different tissue distributions, variable potencies, different receptor specificities, and variable biological functions. Indeed, HRG has been shown to be implicated in developmental processes [12–15], as well as physiological and pathological processes of the nervous system, the heart and in epithelial cells of many different organs {*e.g.*, Schizophrenia (reviewed in [16]), coronary artery disease [17], cancer (see text)} (Table 1).

While HRG proteins encoded by the *HRG-1* and *HRG-2* genes bind to and activate both ErbB3 and ErbB4 receptors, isoforms encoded by the *HRG-3* and *HRG-4* gene only induce activation of ErbB4. HRGs are predominantly expressed in parenchymal organs and in the embryonic central and peripheral nervous system [18, 19] and gene transcripts were found in breast, ovary, testis, prostate, heart, skeletal muscle, lung, liver kidney, salivary gland, small intestine, brain and spleen [3]. Mutation of the HRG-1 gene in the mouse has indicated that it has multiple independent and essential functions in development. HRG-1 knockout mice die during embryogenesis due to severe heart malformation and failure in neuronal cell development [20]. These findings led to a classification of the HRG family members into neuronal and mesenchymal factors, based on their tissue of origin [21]. HRGs are involved in the regulation of cellular proliferation [22–26], differentiation [1,

Table 1. HRG gene products and their expression in different tissues.

HRG gene	Protein encoded by the HRG gene	HRG isoforms	Protein expressed in
HRG-1 type I	HRG / NRG / NDF	HRG-1α1 HRG-1α2a HRG-1α2b HRG-1α2c HRG-1α5 HRG-1β1 HRG-1β2a HRG-1β3 HRG-1β4a HRG-1β5 HRG-1γ	Neural tissue Respiratory epithelia Heart (endocardium) Different organs (epithelial cells)[a]
	ARIA	ARIA-β1 ARIA- β2	Neural tissue Muscle
HRG-1 type II	GGF	GGF2	Neural tissue Pituitary Retinal ganglion Muscle
HRG-1 type III	SMDF	SMDF	Neural tissue Olfactory epithelium Retinal ganglion Heart (endocardium)
HRG-2	HRG-2	HRG-2α HRG-2β HRG-2αυ HRG-2βυ HRG-2α*1 HRG-2α*2	Neural tissue
HRG-3	HRG-3		Neural tissue
HRG-4	HRG-4		Pancreas Muscle

HRG: heregulin; NRG: neuregulin; NDF: Neu differentiation factor; ARIA: acetylcholine receptor-inducing activity; GGF: glial growth factor; SMDF: sensory and motor neuron-derived factor.
[a] Breast*, ovary*, testis, prostate*, heart, skeletal muscle, lung*, liver, kidney, salivary gland, small intestine*, brain*, and spleen.
(* Neoplastic pathologies with HRG expression found in these organs).

27–29], migration [30–34], apoptosis and survival [25, 26, 35–38], angiogenesis [39–41] and cell fate [42–44], depending on the cell type and the HRG isoform. Regarding the diversity and number of HRG genes and isoforms, as well as their wide tissue distribution, it is not surprising that HRG is involved in such a variety of biological outcomes.

Structure of HRG

HRG genes: isoforms and their relevance in human cancer

HRG-1. Human *HRG-1* gene has been mapped to chromosome 8, in the p22-p11 region [45]. This gene contains 21 alternatively spliced exons generating a minimum of 15 HRG isoforms [46], with heterogeneous binding affinities to different ErbB complexes [6]. Based on structural differences in the N terminus, *HRG-1* encoded isoforms can be subdivided into three mutually exclusive groups (Fig. 1). Type I (*e.g.*, HRG; acetylcholine receptor-inducing activity, ARIA) and type II (*e.g.*, glial growth factor, GGF) isoforms contain an immunoglobulin (Ig)-like domain N-terminal to a common epidermal growth factor (EGF)-like sequence and are referred to as Ig-HRGs. While type I proteins contain a cytoplasmic tail as well as an extracellular glycosylation site between the Ig-like and the EGF-like domain, type II proteins lack both of these domains. The third group of HRG-1 proteins belongs to the type III isoforms (*e.g.*, sensory and motor neuron-derived factor, SMDF), containing a cysteine-rich domain (CRD) N-terminal to the EGF-like domain. These HRG variants are referred to as CRD-HRGs (reviewed in [47]). The only exon that is shared by all isoforms is the one encoding the major part of the EGF-like domain, encoding four of the six cysteine residues present in the EGF-like domain.

The EGF-like domain of type I and type II HRG-1 isoforms is preceded by a variable-length sequence that contains one invariant motif, the Ig-like domain, and two mutually exclusive domains, which flank the Ig-like domain on both sides. These are the neuronal

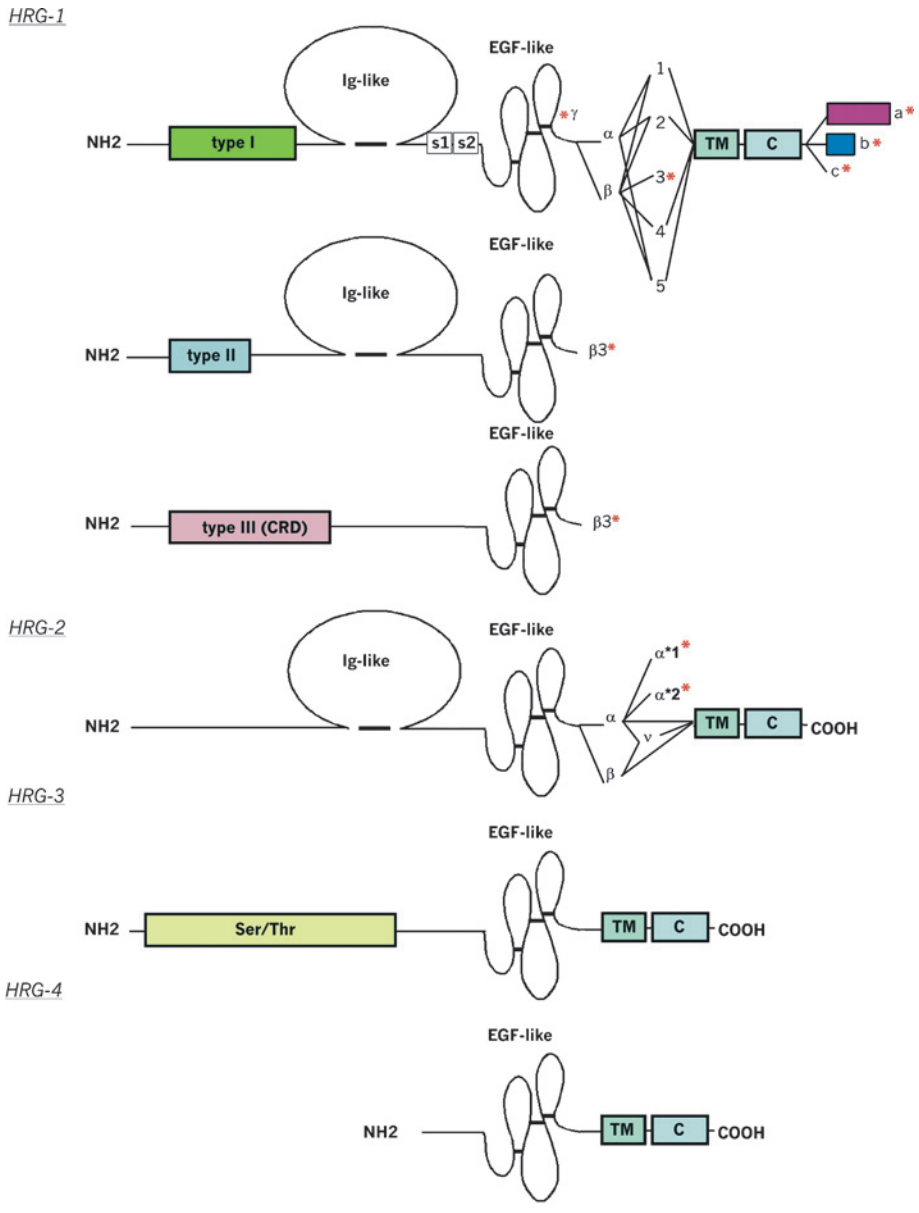

Figure 1. Heregulin (HRG) 1–4 isoform structures of their proproteins: schematic representation of coding sequences. In the *HRG-1* gene, three different start codons may be used, generating type I HRG1 (heregulin, HRG), type II HRG1 (glial growth factor, GGF) and type III HRG1 (schwannoma derived growth factor, SDGF). Ig-like: Ig-like domain, held together by a cysteine bridge; involved in glycosaminoglycan binding. EGF-like: Epidermal growth factor (EGF)-like domain held together by three cysteine bridges; receptor binding and activation. s1 and s2: Spacer sequences; glycosylation site in HRG-1 type I proteins. TM: Hydrophobic transmembrane sequence; anchorage in cytoplasmic membrane. C: Cytoplasmic tail; protein processing and regulation of apoptosis. Ser/Thr: Serine/threonine-rich N terminus of HRG-3 gene splice variants. α, β and γ: Splice variants of the EGF-like domain. 1, 2, 3, 4 and 5: Splice variants of the juxtamembrane region in HRG-1 isoforms. a, b and c: Splice variants of the cytoplasmic tail of HRG-1 type I variants. α*1, α*2: Truncated variants within the juxtamembrane sequence of HRG-2 proteins, due to a translational frameshift. Red asterisks indicate the position of stop codons in HRG-1 and HRG-2 gene splice variants.

cell-specific kringle-domain and the apparently mesenchyme-specific glycosylation spacer domain. Further structural variation is confined to three domains C-terminal to the EGF-like domain and defines the identity of each HRG-1 type I precursor protein [48]. Based on receptor binding affinities and structural differences in the C-terminal portion of the EGF-like domain, HRG-1 type I can be subdivided into α, β and γ isoforms, which differ between the fourth and the sixth cysteine of the EGF-like domain [49]; HRG-1 type II and type III have a β-kind EGF-like domain ending. The α and the β isoforms both bind to the ErbB3 and ErbB4 receptors, with HRG1-β being more potent than HRG1-α and showing higher receptor affinity [50]. While HRG1-γ variant has a longer 5′UTR and a longer 3′UTR than HRG1-α, the protein is truncated after amino acid position 211, resulting in a protein equivalent to the N-terminal 211 amino acids of HRG1-α. Our data suggest that the HRG1-γ isoform does not bind or activate the receptors, as shown by addition of recombinant HRG1-γ to different ErbB-expressing breast cancer cell lines (unpublished data).

The adjacent juxtamembrane processing stretch displays structural heterogeneity that is denoted by a number (isoforms 1–5; isoform 3 contains a stop codon). Lastly, the length of the cytoplasmic tail of type I HRG-1 downstream to an invariant segment that includes the transmembrane domain and 157 amino acids, determines the identity of the isoforms as

a, b or c. Alternatively, there are isoforms that lack the transmembrane and cytoplasmic domains, like HRG1-β3, which has a stop codon C-terminal to the EGF-like domain. These isoforms are not glycosylated and are presumably retained intracellularly. However, their potential functional implication within the cells remains to be determined.

Although the exact mechanism(s) by which HRG-1 induces malignancy may be distinct for different cell types, some general aspects are maintained for the majority of these. Cancer cells that aberrantly produce HRG-1 are likely to use it in an autocrine manner, resulting in constitutive activation of their respective ErbB receptors and the downstream signaling pathways. Consequently, this leads to increased proliferation and invasion of cancer cells, resulting in a more malignant phenotype (described in more detail later in this review). However, HRG-1 is suggested to have a dual function, as it is also described to be involved in the induction of growth inhibition in various tissues, which is related to the ability of HRG to induce apoptosis, differentiation, and cell cycle G2 arrest (reviewed in [35]). Different HRG1-β isoforms were shown to induce apoptosis [51, 52], whereby both the extra-and intracellular domains of HRG-1 are required [53]. Therefore, the difference in the role of HRG-1 in controlling cell proliferation as well as cell fate is mainly due to the presence of different HRG-1 isoforms and the variable expression of ErbB receptors.

HRG-2. Human *HRG-2* gene (also called DON-1 or NTAK) encodes 12 exons, coding for six isoforms [8, 54] and was mapped to chromosome 5, in the q23-q33 region. The HRG-2 mRNA is detected mainly in the nervous system. An alternative splice site within the EGF-like domain in the *HRG-2* gene gives rise to the α and β forms (Fig. 1), followed by a variable region between the EGF-like domain and the transmembrane domain that is either present (ν isoforms) or absent in the HRG-2 transcripts. In addition to transmembrane forms, putative secreted forms have been detected, which show truncation of the proteins in the C-terminal part of the EGF-like domain due to translational frameshift events (α*1 and α*2) [55, 56]. Although the overall structure of HRG-2 proteins resembles that of HRG-1 proteins, the two gene products differ in their amino acid sequence (sequence homology between Ig-like domain: 36%; EGF-like domain: 50%; transmembrane domain: 91%; cytoplasmic domain: 47–89%). Furthermore, several HRG-1-specific domains/exons were not identified in HRG-2 proteins, such as the CRD, the glycosylation domain and the discussed variation in the cytoplasmic tail.

As described for HRG-1, HRG2 proteins are also involved in the induction of cell proliferation as well as in the control of cell differentiation, depending on the expression of the appropriate receptors and the HRG-2 isoforms present [57–59]. Furthermore, HRG-2 has been proposed to inhibit angiogenesis on the basis of its ability to inhibit proliferation of endothelial cells [60]. This effect is not mediated by its EGF-like domain and may be due to receptor-independent mechanisms.

HRG-3. The *HRG-3* gene was mapped to chromosome 10, in the q22-q23 region [61]. Like HRG-2, the expression of HRG-3 mRNA seems to be restricted to the nervous system. In contrast to many HRG-1 family members, the extracellular domain of HRG-3 is devoid of Ig-like or kringle domains. Instead, HRG-3 contains a unique Ala/Gly-rich segment at the N terminus and a mucin-like Ser/Thr-rich region containing abundant sites for O-linked glycosylation, upstream of the EGF motif (Fig. 1) [10]. HRG-3 was found to be expressed in human breast cancer biopsies [62], and is suggested to be a potential regulator of normal and malignant breast epithelial cells *in vivo* [63].

HRG-4. Aside from HRG-4 possessing a HRG-like EGF domain, it shares very little sequence homology to the known HRGs, particularly in the vicinity of the transmembrane domain, a region where the other three HRGs exhibit high primary sequence homology [7]. Unlike other HRGs, which contain a variety of structural motifs, such as an Ig-like domain, a Cys-rich region, or a mucin-like domain, HRG-4 contains no recognizable structural motifs other than the EGF-like domain (Fig. 1). Expression of HRG-4 mRNA has only been detected in pancreas and to a lesser extent in muscle [7]; yet HRG-4 expression was found in breast [63] as well as bladder cancer [64].

HRG motifs

N terminus. Unlike other secreted proteins, which have a classic N-terminal signal sequence that targets the protein to the endoplasmic reticulum for subsequent cell sorting, the HRG proteins do not have this endoplasmic reticulum targeting sequence. Instead, the HRG isoforms display a variety of other sequences, which range from hydrophobic stretches in the N terminus (type II and type III HRG) to signal-anchor sequences within the transmembrane domain (type I HRG) (reviewed in [47]), and which are potentially important for protein trafficking and sorting. Furthermore, the first 23 amino acids of HRG-1 are domi-

nated by charged residues and contain a sequence (KGKKKER; residues 13–18) that closely resembles the consensus sequence motif for nuclear targeting [65], which was first defined for HRG by Holmes et al. [3].

Ig-like domain. The most N-terminal two cysteines of HRG-1 and HRG-2 are linked by a disulfide bond, and the intervening amino acid sequence was identified as an Ig-like domain of the C2 type [65, 66]. Although the Ig-like domain is not directly involved in receptor binding and activation [3, 48], this domain seems to be essential for efficient interaction of the EGF-like domain with ErbB receptors [67] as well as to be important for efficient signal attenuation [68]. Furthermore, the Ig-like domain contributes to the association of HRGs with the extracellular matrix by binding to cell surface heparan sulfate proteoglycans [69], leading to a local enrichment of the growth factor at the site of action [70]. Moreover, we have shown that this sequence motif is implicated in the stabilization of interactions of HRG with intracellular proteins other than the receptors (*e.g.*, hUBC9) [2, 71]. In addition, we identified a second putative nuclear localization sequence (NLS) within the Ig-like domain [71], resembling the consensus NLS sequence K-R/K-X-R/K [72]. Our data demonstrate that either of the two NLS found in HRG is sufficient for nuclear import [71].

Spacer domain. Located N-terminal to the EGF-like domain of HRG-1 type I isoforms, this stretch of 13 amino acids serves as an attachment site for *O*-linked and *N*-linked glycosylation [1]. The region is absent from neuronal HRG isoforms, suggesting that it is transcribed from a separate exon. In addition, the spacer region is N-terminally flanked by a single Ser-Gly dipeptide that is thought to be a potential site for glycosaminoglycan attachment [73]. The spacer domain may serve as a peptide core to adopt a stiff and extended conformation to keep adjacent functional domains, namely the Ig-like domain and the EGF-like domain, exposed and accessible to molecular interactions.

EGF-like domain. This unit is shared by many transmembrane glycoproteins and is defined by six cysteines predicted to fold into a typical structure with three disulfide-linked loops (HRG-1: Cys^{182}–Cys^{196}, Cys^{190}–Cys^{210}, Cys^{212}–Cys^{221}). The EGF-like motif of HRG is essential and sufficient for receptor binding and activation as well as for promoting tumorigenesis [26]. Deletion mutants consisting only of this domain fully retain the receptor binding capacity and the biological activity. Conversely, HRGs lacking this domain do not bind to, and are not able to displace, wild-type HRGs from the receptors [48]. The α/β/γ sequence variation partially alters the amino acid sequence between the fourth and the sixth cysteine residues of the EGF-like domain and completely changes the sequence C-terminal to this motif. The C terminus of the EGF-like domain is responsible for differences in receptor binding affinities and conservation of most of the EGF-like domain confers the same receptor specificity to all HRG proteins. The binding epitope of HRG for the native receptor extends across the beta-sheet in the EGF-like domain and includes residues Leu179, Lys181, Leu209 and Lys211 of HRG [50, 70, 74]. Another study showed that a region consisting of HRG amino acids 177–226 is sufficient both for binding and stimulation of receptor phosphorylation [50]. The β-isoforms bind to the ErbB receptors with an affinity that is approximately tenfold higher than that of the α-isoforms [48]. Therefore, although the α-isoform and the β-isoform share similar protein conformation, they are biologically distinct. It is suggested that this must be related to the unique sequence (*i.e.*, structural) difference in the last disulfide loop in the EGF-like domain and the C-terminal tail (6 amino acids in length) [50]. These data further imply that the receptor-binding domain of HRG molecules resides at the C-terminal region of the EGF-like domain. In contrast, the HRGγ isoform contains the third known sequence variation in the EGF-like domain; this splice variant is encoded by the first 211 amino acids of HRG-1; however, due to a stop codon after the fourth cysteine residue, the EGF-like domain is truncated. Furthermore, HRGγ lacks the transmembrane domain and may therefore act intracellularly.

Considering the function of the EGF-like region in diverse proteins as a motif essential for protein-protein interaction, our work has shown novel non-receptor interaction partners of HRG, binding to its EGF-like motif (*e.g.*, hUBC9, cullin-1, RS cyclophilin, HDAC2) [71].

Juxtamembrane and transmembrane domain. Most HRG precursor proteins are synthesized as membrane-associated growth factors, containing a transmembrane domain that spans the plasma membrane once. Membrane anchoring and membrane topology are influenced by variable hydrophobic transmembrane domains and other structural elements [47, 75]. In general, they are oriented in the membrane in a manner placing the EGF-like domain on the extracellular face of the plasma membrane where it can interact with the respective transmembrane ErbB receptors. Transmembrane HRG proteins can either act in a juxtacrine way [76], activating ErbB receptors

as intact transmembrane proteins, or – after being processed and cleaved as secreted growth factors – act in a paracrine or autocrine way [77, 78]. The hydrophobic membrane-spanning stretch positioned in the C-terminal half of the HRG molecule is flanked at both ends with clusters of basic residues that probably function as transmembrane anchoring sequences. HRG is proteolytically cleaved in the juxtamembrane sequence N-terminal to the transmembrane domain by metalloproteinases [79, 80] to be secreted as a ligand for the ErbB3 and ErbB4 receptors. A putative proteolysis site (Lys-Arg) is common to all transmembrane forms of HRG. This protein-processing step has been shown to involve ADAM proteases, which are major ErbB ligand sheddases [81]. Deletion of the extracellular juxtamembrane region termed linker, decreases cell surface exposure of the mutant proHRG(DeltaLinker), and causes its entrapment at the *cis*-Golgi. Furthermore, cell surface-exposed transmembrane HRG forms retain biological activity, activating ErbB receptors in *trans* and also stimulating proliferation [82]. These data show that the linker is implicated in surface sorting and the regulation of the cleavage of transmembrane HRGs, indicating that this region exerts multiple important roles in the physiology of HRGs. The juxtamembrane domain displays most diversity since five differently spliced HRG-1 variants (1–5) and two HRG-2 isoforms (α*1, α*2) exist. The HRG-1 juxtamembrane subtypes range in size from a single amino acid (isoform 2) to 27 amino acids (isoform 4) and contain in one case a stop codon (isoform 3).

Cytoplasmic tail. Proper release of HRG functional proteins requires its cytoplasmic tail [83, 84]. This relatively long (157 amino acids) and hydrophilic common part of the HRG-1 molecules is rich in serine and threonine residues and is followed by two variable regions (a or b), or by a stop codon (isoform c). The a isoform extends for an additional 217 amino acids, and isoform b extends for an additional 39 different amino acids. The cytoplasmic domains of human, rat and chicken HRG molecules display a high sequence identity (>85 %), which implies a functional role for this long domain. Indeed, one HRG isoform, HRG-1β2b, was isolated in an *in vitro* screen for dominant, apoptosis-inducing genes [52]. The intracellular region of the HRG-1 precursor was sufficient for the induction of apoptosis, independent of ErbB receptor. The HRG-2, -3 and -4 proteins also encode an intracellular cytoplasmic tail, the function of which has not been defined yet.

Subnuclear localization domain. HRG proteins were previously shown to translocate to the nucleus [3, 85]. We and others have shown that nuclear HRG accumulates in specific intranuclear domains [71, 86]. Detailed analysis of the structural requirements showed that the first 79 amino acids of HRG-1 are necessary and sufficient to direct the protein to nucleoli and nuclear speckles [86]; our data further identified an additional domain (amino acids 113–148, C-terminal of the Ig-like domain), which is also required for subnuclear localization of HRG-1 proteins [71]. Within this first dot-forming sequence we identified a putative consensus sequence for SUMOylation [87], (I/L)KXE (amino acids 34–45) [71], which has been implicated in the regulation of protein targeting for nuclear import [88]. However, as neither SUMOylation of HRG nor the functional implication for nuclear translocation of HRG have been studied further, this leaves an interesting open question regarding the mechanism defining nuclear import of HRG. Indeed, although processed HRG proteins have a size of around 45 kDa, which is below the diffusion size for nuclear pore complexes, our results suggest that either an active import mechanism or an active nuclear retention mechanism exists for HRG proteins [71].

HRG acts as a ligand for the ErbB3 and ErbB4 receptors

Most HRG isoforms are synthesized as large transmembrane precursor proteins, with the EGF-like domain connected to the transmembrane domain by the juxtamembrane linker, which is susceptible to proteolytic cleavage by metalloproteinases thereby releasing the HRG ectodomain [79, 80]. Processing of the pro-HRG at both the N and the C termini generates the secreted molecule [50, 81] that may bind to nearby ErbB receptors, acting in an autocrine or paracrine way (type I and II HRG mainly) [81, 82]. Alternatively, intact transmembrane HRG proteins may directly activate ErbB receptors, leading to a juxtacrine type of signaling (mainly type I and type III HRG, in addition to other isoforms) [76]. Furthermore, as the Ig-like domain of HRG proteins has been shown to bind to heparin sulfate proteoglycans and other highly charged glycosaminoglycans [70], localized at the cell surface and extracellular matrix, this may provide a way to limit diffusion as well as to enrich HRG proteins at their site of action. It has been reported that heparan sulfate binding may alter the biological activity of growth factors [89].

The ErbB family of receptors and their ligands are described as a "signaling network", including an input layer (ligands, receptors, transactivators); a signal-processing layer (downstream intracellular adaptor

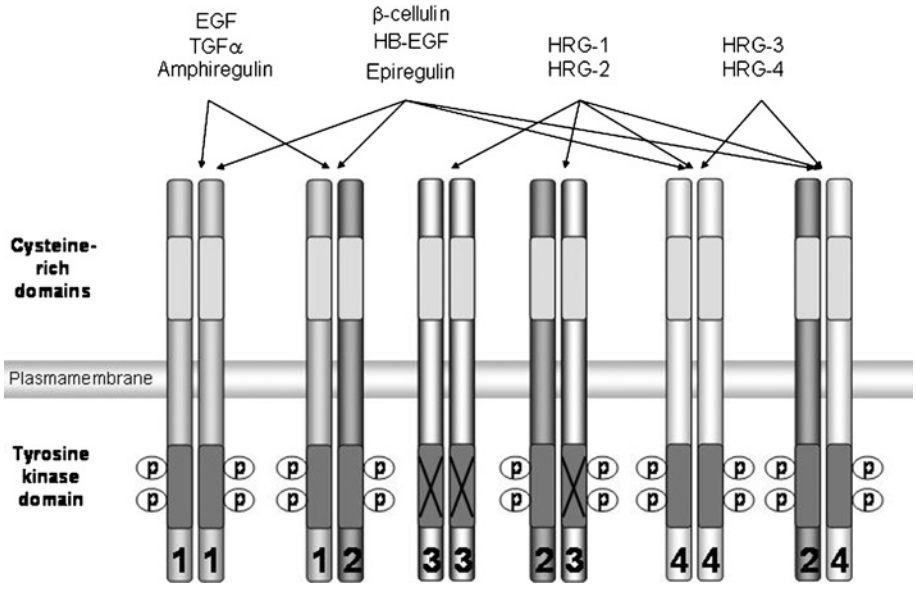

Figure 2. ErbB family of transmembrane receptor tyrosine kinases act as receptors of the EGF family of growth factors. The cysteine-rich domain in the extracellular domain of ErbB receptors acts as a ligand-binding site. Ligand binding induces either ErbB receptor homo- or heterodimerization, followed by transactivation of the intrinsic kinase domain located in the cytoplasmic tail. ErbB2 is an orphan receptor with no known ligand; however, it acts as preferred heterodimerization partner of all the other ErbB receptors. ErbB3 has an impaired kinase activity and requires dimerization with other ErbB members to activate its signaling potential.

proteins, signaling cascades, transcription factors); and an output layer (biological consequences of ErbB-ligand interactions, such as proliferation, differentiation, survival) [90]. Each ErbB receptor seems to be responsible for the control of amplitude and duration of cellular biochemical reactions [91]. Like with neural networks, there is convergence and divergence between the different layers as well as lateral interactions within each of the layers.

The ErbB family of receptor tyrosine kinases is expressed in a variety of tissues of epithelial, mesenchymal and neuronal origin, where they play a fundamental role in mediating proliferation, differentiation and survival of normal cells [9, 92]. These receptors have also been implicated in angiogenesis, cell motility and invasion [93], thereby playing a pivotal role in human malignancies [94]. The ErbB family consists of four closely related transmembrane receptors: ErbB1 (also termed EGFR or HER1), ErbB2 (also termed HER2 or Neu), ErbB3 (also termed HER3) and ErbB4 (also termed HER4) (Fig. 2) [4, 95–98]. Structurally, these receptors consist of an N-terminal extracellular ligand binding domain, a single transmembrane domain and a large cytoplasmic tail with a tyrosine-rich C-terminal region and a kinase domain [90, 92, 99]. While EGFR serves as a receptor for EGF, TGFα, HB-EGF, amphirgulin, betacellulin and epiregulin, ErbB2 is an orphan receptor with no known high-affinity ligands. ErbB3 and ErbB4 act as receptors for HRG proteins [6]; in addition, ErbB4 serves as a receptor for betacellulin. The tyrosine kinase activity of the ErbB receptors is induced upon ligand binding, leading to receptor homo- or heterodimerization and subsequent receptor transphosphorylation and activation of downstream signaling events [100]. In principle, phosphorylation of growth factor receptors can occur either in *cis* (within a receptor) or in *trans* (between receptors). *trans*-phosphorylation occurs between EGFR/ErbB2 dimer partners after stimulation with EGF [101] and between ErbB2 homodimer partners [102]. Despite the lack of kinase activity of ErbB3, the phosphorylation of ErbB3 in human cancer cells can be upregulated by ligands, indicating that *trans*-phosphorylation occurred [103]. Although *cis*-phosphorylation was thought to be occurring in ErbB2, as well as in ErbB3 co-expression cells [35], it was not fully clear whether or not ErbB2 can be *cis*-phosphorylated in a heterodimer of ErbB2/ErbB3 or ErbB2/ErbB4. However, a recent study nicely demonstrates that *trans*-phosphorylation, but not *cis*-phosphorylation occurred between ErbB2/ErbB3 and ErbB2/ErbB4 heterodimer partners by HRG-1 stimulation [104].

Signal diversity after receptor activation is determined through differences in the tyrosine residues that undergo phosphorylation [105]. Which sites are autophosphorylated and hence which signaling proteins are engaged, is determined by the identity of the ligand as well as by the heterodimerization partner [106]. Furthermore, the subsets of signaling molecules that couple to an activated receptor undergo time-dependent changes, suggesting that ErbB receptor phosphorylation is not static. The fine tuning of this cascade most probably governs the decision of differentiation or proliferation upon growth factor stimulation [107].

All human ligand-binding ErbBs adopt a tethered conformation in the absence of ligand. The presence of this tethered state, in which the dimerization arm is sequestered, strongly suggests that ErbB3 and ErbB4

undergo conformational changes following HRG binding, allowing receptor dimerization and activation [108]. The tethered contact masks the dimerization loop thereby presenting a barrier to the formation of a high-affinity ligand-binding site. The orphan receptor ErbB2 adopts a conformation very similar to the ligand-activated form of ErbB1, in which the dimerization loop is constitutively exposed [109, 110]. This conformation of ErbB2 appears to explain its readiness to partner with each of the other ligand-bound ErbBs [92].

Binding of HRG to its receptors results in the autophosphorylation of ErbB4 and the subsequent formation of ErbB4 homodimers as well as of ErbB4-ErbB2 and ErbB3-ErbB2 heterodimers [49, 57, 111]. It has to be noted that ErbB3 is not a substrate for HRG-activated ErbB4 [112]. As ErbB3 has no kinase activity (kinase dead receptor) [113], it depends on heterodimerization for its activation after ligand binding [114, 115]; indeed, ErbB2 acts as the preferred heterodimerization partner of the other ErbB family members, including ErbB3. The increased potency of ErbB2-containing heterodimers can be attributed to three reasons: first, the heterodimers are characterized by a relatively slow rate of ligand dissociation [116]; second, unlike ErbB1, whose rate of ligand-induced endocytosis is rapid, ErbB2 is a slowly internalizing receptor [117]; and third, ErbB2 co-expression increases the fraction of high-affinity receptors at the cell surface [118]. Thus, signaling by ErbB2-containing receptor dimers is relatively prolonged and results in enhanced activation of signaling pathways. Indeed, ErbB2-ErbB3, which seems to be the most potent heterodimer, was shown to be highly mitogenic [57, 111].

HRG proteins are the only ligands capable of activating all four ErbB receptors by heterodimerization, which allows a broader range of biological effects induced by these ligands. It has been postulated that the different cellular responses to HRG are dependent on the complement of ErbB receptors in a particular cell type and the presence of other ErbB ligands in the microenvironment [100]. Apparently, ErbB3 is associated with proliferation, whereas ErbB4 correlates with a differentiated phenotype and the two HRG receptors play distinct, rather than redundant, developmental and physiological roles [119]. Furthermore, transactivation of other receptors may contribute to the actions of HRG [49], as shown in the case of the progesterone receptor [120].

HRG in human cancer

The interaction between the ErbB tyrosine kinase receptors and their ligands plays an important role in tumor growth. Aberrant ErbB receptor activation may be the result of receptor truncation or mutation, association with other cell-surface proteins, transactivation via other receptors or the presence of autocrine loops [121], and has been correlated with cancer development and progression [90, 122] of a variety of tumor types [123]. Disrupting this autocrine loop may provide an important therapeutic measure to control cancer cell growth [124]. The vast majority of publications describing the role of HRG in cancer development focus on the HRG-1 isoforms. However, the other HRGs may also play a role in malignancy, as HRG-2, HRG-3 and HRG-4 have been shown to stimulate cell proliferation, provided the cells express the appropriate receptors [62, 125]. In the following section of this review, I focus on HRG-1 proteins, and unless indicated explicitly otherwise, the terms "heregulin" and "HRG" refer to HRG-1 proteins.

While the action of HRG *in vivo* is typically paracrine, being expressed in mesenchymal tissues adjacent to epithelia, epithelial tumors frequently show gain of expression activating an autocrine loop and/or increased sensitivity to paracrine signaling. It has been shown in different human cancer cells that the relative level of ErbB2, ErbB3 and ErbB4 can modulate the response to HRG, determining whether the response is stimulatory or inhibitory [126]. Co-expression of ErbB2 and ErbB3 reconstitutes a high-affinity receptor for HRG, capable of potent mitogenic signaling [127]. Various studies have shown that HRG-stimulated ErbB3 activation in breast cancer can induce tumor progression, invasion and metastasis [128–131]. While the ErbB3 receptor seems to have direct implication in tumor formation by HRG, the function of ErbB4 in human malignancies remains unclear. Activation of ErbB4 by HRG can either result in proliferation or differentiation, most probably due to the range of signals generated by ErbB4 homodimers *versus* heterodimeric complexes with other ErbB members. Indeed, there is evidence that ErbB4 expression correlates with a more differentiated tumor grade, longer survival, and positive prognostic indicators [132–136]. One way HRG/ErbB4 may impair cellular proliferation or promote differentiation is through induction of a cell cycle delay in the early phase of mitosis and increased expression of the tumor suppressor protein BRCA1 [137], potentially delaying tumor formation and progression. Moreover, HRG induction of apoptosis is directly correlated with decreased MAPK activity, increased JNK activity

resulting in down-regulation of the ErbB2 [138] and the stabilization of p53 [139].

Tumor types

HRG has been shown to be involved in different types of cancer: breast, ovarian, endometrial, colon, gastric, lung, thyroid, glioma, medulloblastoma, melanoma as well as head and neck squamous carcinoma. In most of these tumor types, HRG regulates growth, invasion and angiogenesis through either overexpression or activation of an autocrine or paracrine loop. Autocrine HRG may give rise to constitutively activated ErbB2 and ErbB3, protecting these tumors against apoptosis and generating growth factor independence.

Breast cancer. The growth and progression of breast carcinomas are regulated by a plethora of signals mediated by growth factors and steroid receptors. About 60% of the human breast tumors express estrogen receptor (ER) and are characterized by a better prognosis and response to endocrine treatment. Unfortunately, at some point, most of the initially responsive patients will fail the endocrine treatment and will develop more aggressive tumors. The more aggressive tumors have been correlated with up-regulation of ErbB2. Co-expression of ErbB2 and ER in breast cancer cells was shown to confer resistance to endocrine therapy. HRG was demonstrated to be required in the morphogenesis and differentiation of the mammary gland [140]. Importantly, HRG is overexpressed in about 30% of breast tumors that do not overexpress ErbB2 and co-expression of HRG contributes to ErbB2/ErbB3 activation in an autocrine or paracrine fashion, inducing malignant transformation of mammary epithelial cells [124, 141]. Various studies suggest that HRG is involved in the acquisition of a hormone-independent phenotype [131] and anti-estrogen resistance of breast cancer [142–144], inducing a more aggressive phenotype. This lead to the proposal of HRG as a relevant prognostic/diagnostic factor in breast cancer [145]. In human breast carcinomas, a higher percentage of ER-negative tumors express HRG compared to ER-positive tumors [146]. HRG can overcome the protective effects of ER and at least a component of this appears to be due to repression of estrogen-responsive element (ERE)-dependent transcription [147]. Recent studies have demonstrated that HRG up-regulation alone is sufficient for the development of mammary tumors and promotion of metastasis, even in the absence of estrogen stimulation and independent of ErbB2 overexpression, inducing hormone independence and anti-estrogen resistance via increase of MMP-9 and VEGF in an autocrine manner [128]. HRG promotes the invasive behavior of breast cancer cells also by regulating the actin cytoskeleton, thereby inducing cell motility [148]. Blockage of HRG expression suppresses the aggressive phenotype by inhibiting tumor growth and metastasis [129], highlighting the role of HRG in tumor progression. It has to be mentioned that several reports show an anti-proliferative role of HRG, promoting cell differentiation or apoptosis [51, 149–151], contradicting other studies showing proliferative and mitogenic effect of HRG. Although it is currently unclear what factors determine whether HRG acts as a mitogen or promotes cell death and differentiation, there are studies showing that the biological response to HRG seems to depend directly on the level of ErbB2 expression in breast cancer cells [27, 29, 51, 149, 152–154].

Ovarian cancer. HRG expression was found in the majority of ovarian carcinomas and cell lines, and a growth-stimulatory, rather than a growth-inhibitory role for HRG has been described in human ovarian epithelial cells, with a potential for autocrine regulation of cell growth. The expression level of ErbB1, ErbB2, ErbB3 and ErbB4 can profoundly affect HRG response, with ErbB3 and ErbB2 levels exhibiting the maximum association with growth stimulation and ErbB4 having a more complex role [22, 155].

Endometrial cancer. The human endometrium is perhaps the most dynamic tissue in the body that undergoes cyclical proliferation, differentiation, and shedding in response to the female sex hormones, and persistent and prolonged estrogenic stimulation is a well-known risk factor of endometrial carcinoma. While ErbB receptors and their ligands have been shown to be involved in endometrial maturation, overexpression of the receptors ErbB3 and ErbB4 and decrease in HRG1α expression has been described to induce endometrial adenocarcinoma [156].

Colon and gastric cancer. Although rarely overexpressed, ErbB2 is widely expressed at normal levels in colon cancers, suggesting a possible growth-regulatory role for this ErbB family member in this disease. Indeed, HRG co-expression and autocrine activation of ErbB2 through dimerization with ErbB3 has been described in colon carcinoma cells, and autocrine HRG activity was responsible for growth factor independence [38], providing the cells with a cell survival mechanism during growth factor and nutrient depletion. Although HRG, ErbB3 and ErbB4 mRNA were detected in esophagus, stomach and duodenum [157], very little is known about the function of HRG

in the gastrointestinal (GI) tract. There are data suggesting that HRG may affect epithelial cell proliferation through mesenchymal-epithelial interaction in the gastric mucosa [158]. Moreover, in gastric cancer, mRNA for ErbB4 was significantly overexpressed. These findings suggest that HRG and its receptors may be physiologically significant in the human upper GI mucosa, especially in duodenum, and that ErbB4 may contribute to the growth of gastric cancer.

Lung cancer. Expression of ErbB receptors and HRG is differentially expressed in normal bronchial epithelial and non-small cell lung carcinoma (NSCLC) cell lines, and it was proposed that a constitutive activation of ErbB2, ErbB3 and ErbB4 receptors could be induced by HRG via an autocrine loop mechanism in human lung carcinogenesis [159, 160]. While *ErbB2* gene amplification or 3+ staining by immunohistochemistry is only present in 2–5 % of patients with NSCLC, ErbB2 expression detectable by immunohistochemistry is present in about 25 % of NSCLCs [161, 162]. Furthermore, ErbB2 has been shown to be mutated in a subset of NSCLC [163, 164] and a recent study has shown that somatic mutations and overexpression of wild-type ErbB2 found in NSCLC leads to oncogenic transformation in a murine NSCLC tumor model [165]. These data suggest that the ErbB2 receptor plays an important role in the development of NSCLC, independent of ligand binding. The EGF receptor is detected by immunohistochemistry in 50–80 % of NSCLCs and is activated by amplification and/or mutations in a subset of these tumors [166, 167], providing evidence that these cancers also require EGFR activity for the maintenance of critical intracellular survival and growth signaling pathways. Furthermore, it is suggested that EGFR works in concert with other ErbB family members, particularly ErbB2 and ErbB3, to activate these signaling pathways in lung cancers (reviewed in [168]).

Prostate cancer. While ErbB1, ErbB2, and ErbB3 expression was observed in prostate carcinoma cell lines, ErbB4 was absent and HRG was expressed only in an immortalized, non-transformed prostate epithelial line [169]. Furthermore, HRG inhibited prostate cancer cell growth and induced an epithelial-like morphological change, mimicking differentiation. Immunohistochemical studies in clinical prostatectomy specimens demonstrate absence of significant HRG expression in prostate cancer, whereas it is expressed in 100 % of the stroma, 100 % of basal epithelial cells, and 58 % of luminal cells in normal and benign hyperplastic prostatic tissue. These data suggest that HRG may be a paracrine differentiation factor involved in normal adult prostate physiology and that functional loss of the HRG/ErbB paracrine loop may be an early event associated with prostate tumorigenesis [170]. However, another study found overexpression of HRG and ErbB3 in a panel of human prostate cancer, suggesting a potential autocrine loop between HRG and ErbB3 in human prostatic adenocarcinoma [171]. Indeed, recent data show that activation of ErbB2 and ErbB3 by HRG enhances androgen receptor (AR) transactivation and growth of recurrent prostate cancer cells in the absence of hormone [172].

Papillary thyroid cancer. Immunohistochemical analysis revealed increased levels of HRG in both primary thyroid tumors and lymph node metastasis, as compared to normal thyroid tissue. However, no association was found between HRG protein expression and clinical parameters. The vast majority of patients showed nuclear immunostaining of HRG in the papillary carcinomas but not in the normal adjacent tissue. Overexpression and nuclear localization of the HRG were not associated with the expression of ErbB receptors; this may reflect an unknown mechanism of HRG action, possibly independent of the ErbB receptor system [173].

Glioma. HRG is widely expressed in neurons and glia as well as in gliomas [174], and has been shown to be implicated in a number of developmental events in neuronal cells, including enhanced survival, mitosis, migration and differentiation. Glioma cells may use autocrine or paracrine HRG signaling to enhance cell survival, rather than cell proliferation [36, 175]. Moreover, HRG is suggested to play an important modulatory role in glioma cell invasion, increasing cell motility through activation of focal adhesion kinase [33, 176]. However, in glioma, EGFR is frequently overexpressed, which is often associated with gene amplification. Furthermore, activation mutation of EGFR has been commonly observed in glioma. In contrast, other EGFR family members may be present but are not commonly amplified, overexpressed, or mutated in gliomas (reviewed in [177]). This suggests that these cancers mainly depend on EGFR signaling for the maintenance of survival and growth signaling pathways.

Medulloblastoma. ErbB1, ErbB3, ErbB4, and HRG display specific temporal and topographical distribution in the cerebellum and normal ErbB/HRG signaling is likely to be mediated by ErbB4. In contrast, ErbB2, which is expressed in 86 % of medulloblastomas, could not be detected at any stage of cerebellar

development. This suggests the formation of a HRG/ErbB2/ErbB4 autocrine loop as an important factor in medulloblastoma tumorigenesis. Indeed, HRG expression is observed in 87% of medulloblastoma primary tumors (cytoplasmic and nuclear), with the greatest expression levels occurring in tumors with high ErbB2 and ErbB4 receptor co-expression. Furthermore, the expression of all three components of the proposed autocrine loop was significantly related to the presence of metastases at the time of diagnosis [178].

Melanoma. The HRG/ErbB system is functional in melanocytes and in the majority of melanoma cell lines, leading to growth stimulation. ErbB2 overexpression has been associated with transformation and invasion of malignant melanoma. Loss of the full-length ErbB4 receptor in melanoma cells suggests switches in ErbB signaling pathways, perhaps by ErbB3 heterodimer formation, with ErbB2 contributing to the dysregulation [179]. Lack of stimulation by HRG in some melanoma cell lines is due to the loss of expression of ErbB3 protein or to a severely impaired ErbB2 activation. In contrast, the aberrant expression and secretion of HRG by melanoma cells may serve as an autocrine and/or paracrine signal, promoting cell growth and/or migration [180]. Therefore, multiple deregulations of the HRG/ErbB system found in human melanoma cell lines have been suggested to control proliferation and migration of melanoma cells.

Head and neck squamous carcinoma. Different HRG isoforms induce distinct growth regulatory effects on cultured keratinocytes, through direct interaction with ErbB3 [181]. HRG may function as a paracrine mediator controlling epidermal homeostasis [182] as well as directing initial epidermal migration during cutaneous tissue repair [183]. The role of HRG as a motility factor for keratinocytes in epidermal and mucosal wound healing parallels their motility and growth induction capacity in carcinogenesis [184]. Multiple ErbB ligands differentially modulate proliferation, invasion and expression of matrix metalloproteinases in human head and neck squamous carcinoma cells (HNSCC) *in vitro* [185], and ErbB receptor signaling is suggested as a critical element in the pathogenesis and progression of HNSCC, emphasizing the role of autocrine ligand production [186].

Pancreatic cancer. The EGF family of ligands and receptors plays an important role in the pathogenesis of pancreatic ductal adenocarcinoma and contributes to its aggressiveness. *In vivo*, HRG is up-regulated in pancreatic cancer tissues and localized predominantly in the cancer cells. High HRG-β levels but not HRG-α levels are associated with decreased patient survival [187]. Interestingly, HRGs can also induce pancreatic cancer cell growth without the presence or activation of ErbB3 and ErbB4, pointing to a receptor-independent role in pancreatic tumor development.

Regulation of HRG in human cancer

Although relative little is known about the mechanisms involved in the regulation of HRG expression, there are various mechanisms that have been discussed recently. Overexpression of HRG has been found in different human cancers [156, 171, 173], indicating the potential of autocrine regulation. Different growth factors [140, 188] and hormones [28, 189] have been shown to up-regulate HRG expression in different cellular systems. Furthermore, cross-induction, *i.e.*, up-regulation 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 proteins are often co-expressed, leading to a sustained mutual co-amplification mechanism [186]. Other oncogenes may also induce HRG expression in certain tumor cell lines [190, 191]. As HRGs are synthesized as transmembrane precursor proteins, they have to undergo proteolytic cleavage; this process has been linked to multiple metalloproteases of the matrix metalloprotease (MMP) and a disintegrin and metalloprotease (ADAM) family. This shedding can be activated by various physiological stimuli, linked to the enhancement of metalloprotease activity (reviewed in [192]), which regulates the availability of HRG in the extracellular matrix. Indeed, targeting ADAM-mediated ligand cleavage has been shown to inhibit ErbB3 and EGFR pathways in NSCLC [81]. Furthermore, rearrangements of the HRG gene have been implicated in cancer development. Rearrangement and co-amplification of the 8p12 and 11q13 chromosomal regions are found in a significant proportion of breast cancers giving rise to a mutant HRG fusion gene (called gamma-HRG), which shows deregulated signaling through the ErbB pathway [193, 194]. Furthermore, novel HRG gene rearrangements have been described in breast cancer, which are associated with poor prognosis [195]. Subcellular localization of HRG may also be involved in the regulation of HRG activity, as nuclear HRG staining has been shown in medulloblastoma [178], in papillary thyroid carcinomas but not in normal thyroid tissue [173], as well as in ductal carcinoma *in situ* of the breast, where the expression correlates with the tumor grade [196]. Although the significance of intranuclear HRG ex-

pression remains to be determined, it suggests a novel mechanism of action for some of the HRG isoforms. We and others have clearly demonstrated that HRG localizes to specific subnuclear domains, independent of receptor binding [71, 86]. This supports the idea that secretion and subsequent cell surface receptor binding of HRG are not a prerequisite for nuclear translocation of HRG, and that non-secreted ligands may have highly specific activities in defined nuclear compartments, such as the nucleoli and SC35-positive nuclear speckles, involved in ribosomal biogenesis and pre-mRNA splicing [86]. Further evidence for novel nuclear as well as cytoplasmic functions of HRG arises from our recent study identifying HRG protein interaction partners that are expressed in the cytoplasm or in the nucleus [71], and which may be involved in further regulating HRG activity and function during tumorigenesis.

Nuclear localization has been shown not only for HRG proteins but also for the ErbB receptors [197–202]. HRG-mediated activation of ErbB receptors is shown to result occasionally in the nuclear localization of full-length or cleaved receptors, suggesting direct, receptor-mediated signaling [203, 204]. Indeed, EGFR, ErbB2, and ErbB4 are proposed to contain transactivational activity, functioning as transcriptional cofactors to activate gene promoters, and products of these genes are shown to be involved in tumorigenesis and tumor progression [201, 205–207]. However, our understanding of the functional importance of nuclear receptors is very limited and controversial. EGFR is shown to be translocated to the nucleus as an intact receptor; however, the underlying mechanism and functional relevance remains unclear (reviewed in [203, 208, 209]). There are other studies showing that intact receptor tyrosine kinases may be translocated to the nucleus in a ligand-dependent way; yet these data do not clearly support an important signaling role of intact receptor tyrosine kinases in the nucleus (reviewed in [210]). By contrast, ErbB4 is shown to get proteolytically cleaved by a dual-protease system (TACE/γ-secretase) after binding to HRG [203]. The free cytoplasmic tail of ErbB4 translocates to the nucleus as a presumably active form [199], which is suggested to be implicated in the regulation of gene transcription and cell fate [211].

HRG regulates gene transcription. Several HRG-responsive target genes are known to regulate malignant tumor progression. Increased *de novo* formation of vascular systems contributes to tumor progression, supplying the tumor with oxygen and growth factors but also providing a system by which tumor cells can spread to other tissues. Up-regulation of VEGF, CYR61 and Hif-1α by HRG may have direct implication in HRG-stimulated induction of angiogenesis [212–214]. Other proteins that were up-regulated following HRG treatment include MMP-9, stromelysins, collagenases, adhesion molecule 1 as well as urokinase plasminogen activator and its receptor [215, 216]. These proteins play a potential role in HRG-mediated invasion. Furthermore, HRG induces the expression of transcription factors, a mechanism that may constitute an important way of HRG-mediated regulation of a variety of growth-regulating cellular genes [217–219]. Heat shock protein-70 (Hsp70) is another of the HRG-inducible gene products found in human breast cancer cells; since Hsp70 acts as a molecular chaperone with cell survival function, these findings suggest that stimulation of Hsp70 expression is a potential mechanism of protein redistribution in growth factor-activated cells [220]. Heat shock proteins (HSPs) play a key role in the protection of cells from apoptosis and the mediation of anchorage independent growth by HRG, supporting a role for HRG-induced HSP expression in tumor progression [221]. HRG-induced stimulation of ErbB receptors may activate downstream signaling events ultimately leading to control of gene expression. In contrast, although no DNA-binding domain has been described thus far for HRG, this protein may regulate transcription indirectly by recruiting cofactors essential for transcriptional control. Indeed, in a yeast two-hybrid screen, we found that HRG interacts specifically with several proteins implicated in transcriptional regulation [71], supporting the hypothesis that nuclear HRG may be directly implicated in transcriptional control. In addition, we have shown that HRG interacts with endogenous HDAC2, a well-known enzymatic transcriptional corepressor, and shows transcriptional regulation activity in a reporter gene assay [222].

Angiogenesis. MMP-9 plays important roles in tumor invasion and angiogenesis. Secretion of MMP-9 has been reported in different tumor types. HRG not only acts as a mitogenic factor, it further activates MMP-9 [130] and induces VEGF expression and secretion in cancer cells [4], thereby potentially regulating tumor angiogenesis. HRG also leads to an angiogenic response that is independent of VEGF *in vitro* and *in vivo*, involving HRG-induced rapid calcium fluxes, receptor tyrosine phosphorylation, and cell proliferation of endothelial cells [39]. αvβ3 integrin overexpression in tumor-associated vasculature is a marker of poor prognosis. HRG is shown to regulate αvβ3 levels as well as αvβ3-triggered signaling in highly invasive breast cancer cells [223], thereby inducing cell proliferation and survival.

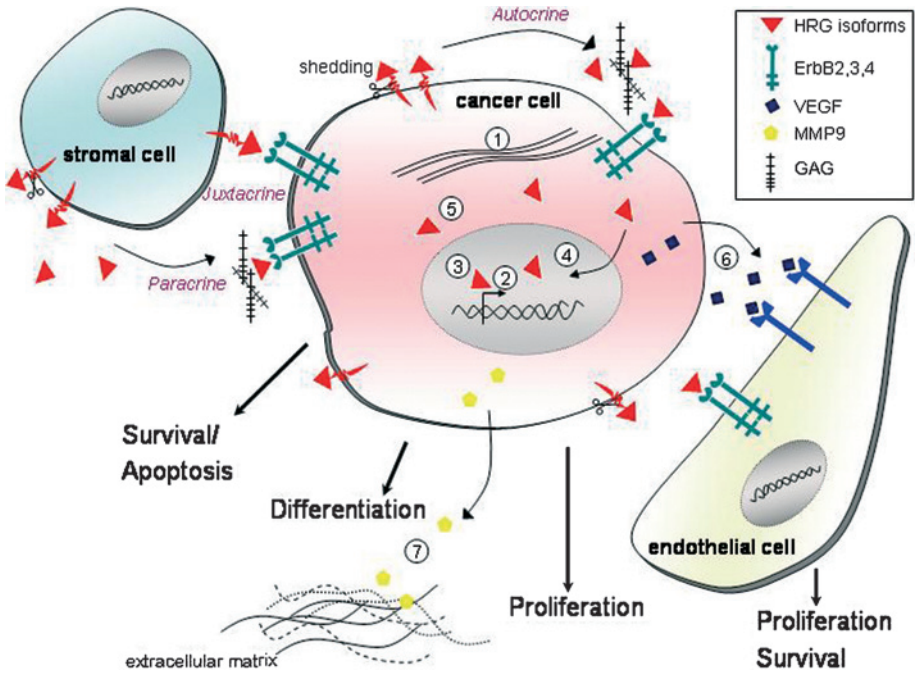

Figure 3. Simplified scheme of HRG-induced tumorigenesis. HRG regulates different pathways involved in cancer development, either in a paracrine, juxtacrine or autocrine way. (1) Regulation of cytoskeletal rearrangements implicated in cancer cell migration and invasion. (2) Regulation of gene expression by inducing transcription factor activity, or (3) by directly acting on gene transcription. (4) Accumulation within subnuclear domains (functional role yet to be determined). (5) Interaction with cytoplasmic proteins (mechanism yet to be studied). (6) Induction of VEGF production and secretion in cancer cells as well as direct activation of ErbB receptor on endothelial cells, inducing angiogenesis. (7) Induction of proteases implicated in the degradation of the extracellular matrix (*e.g.*, MMP-9), thereby regulating cancer cell invasion. Local enrichment of HRG at site of action through binding to glycosaminoglycan (GAG) chains. HRG activity ultimately leads to proliferation and survival of cancer cells as well as of endothelial cells, thereby promoting tumor growth and metastasis.

Invasion. The exposure of cells to growth factors has been shown to induce cytoskeletal reorganization, leading to stimulation of cell motility and invasion. In this context, HRG was shown to promote motility and invasiveness of cancer cells through the regulation of autocrine motility factor expression [224]. As the process of cell migration must involve dynamic changes in the formation of new focal adhesions at the leading edge and dissolution of preexisting focal points, several studies have demonstrated the potential role of HRG in the regulation of paxillin, a major component of focal adhesion [225, 226], during HRG-induced cell shape alterations and motility. In addition, HRG enhances the formation of lamellipodia, membrane ruffles, stress fibers and filopodia, which is accompanied by increased cell migration [227], through regulation of PAK-1 via PI-3 kinase. Tyrosine-phosphorylated ErbB3 is able to directly couple to PI-3 kinase, a lipid kinase involved in the proliferation, survival, adhesion and motility of tumor cells [228], and ErbB3-dependent signaling through ErbB3/ErbB2 heterodimers is shown to contribute to metastasis through enhancing tumor cell invasion and intravasation *in vivo* [229]. For cells to invade the neighboring extracellular matrix, a regulated degradation of matrix proteins is required. Multiple signaling pathways have been described to be involved in the activation of MMP-9 by HRG in human breast cancer cells [130], leading to enhanced cancer metastasis. In addition, mucins provide a protective barrier for epithelial surfaces, and their overexpression in tumors has been implicated in malignancy. Muc4, a transmembrane mucin that promotes tumor growth and metastasis, physically interacts with the ErbB2 receptor tyrosine kinase and augments receptor tyrosine phosphorylation in response to the HRG [230].

Conclusion

The HRG / ErbB system is involved in various physiological events, where it plays important roles in developmental processes and the maintenance of tissue homeostasis. HRG acts not only as a mitogenic factor for certain cells, it is also implicated in the regulation of cell differentiation and the induction of apoptosis. There is a tightly regulated balance between the expression of the ligands and their receptors in these tissues, and deregulation is involved in malignant tumor progression. HRG-induced cell transformation is the result of aberrant signaling

events, resulting from either constitutive receptor activation or the presence of autocrine or paracrine loops involving HRG. There are different mechanisms of HRG-induced tumorigenesis (Fig. 3): (I) activation of angiogenesis and invasion; (II) HRG-mediated regulation of gene transcription inducing expression of proteins important in cancer progression; (III) overexpression of HRG leading to activation of an autocrine loop stimulating proliferation and survival; and (IV) subcellular localization of HRG (role in tumor formation yet to be defined). Since HRG proteins exert their action mainly through receptor binding, a targeted therapeutical approach to inhibit HRG-induced tumorigenesis may involve inhibition of receptor activation through either the use of monoclonal antibodies, interfering with receptor heterodimerization, or the use of selective small molecule inhibitors, inhibiting receptor phosphorylation and transactivation.

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