

“Shedding” light on HER4 signaling in normal and malignant breast tissues

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ABSTRACT

Receptor Tyrosine Kinases of the Epidermal Growth Factor Receptor Family play a pivotal role as drivers of carcinogenesis and uncontrolled cell growth for a variety of malignancies, not least for breast cancer. Besides the estrogen receptor, the HER2 receptor was and still is a representative marker for advanced taxonomic sub-differentiation of breast cancer and emerged as one of the first therapeutic targets for antibody based therapies. Since the approval of trastuzumab for the therapy of HER2-positive breast cancer in 1998 anti-HER2 treatment strategies are being modified, refined, and successfully combined with complementary treatments, nevertheless there is still potential for improvement.

The HER2 relatives, namely HER1 (i.e., EGFR), HER3 and HER4 share a high degree of molecular homology and together form a functional unit for signal transmission. Under regular conditions, receptor coexpression patterns and receptor interaction represent key parameters for signaling robustness, which ensures cellular growth control and enables tissue differentiation. In addition, treatment efficiency of e.g., an anti-HER2 targeting is substantially determined by the expression pattern of HER receptors on target cells.

Within the receptor family, the HER4 plays a particular role and is engaged in exceptional signaling activities. A favorable prognostic impact has been attributed to HER4 expression in breast cancer under specific molecular conditions. HER4-specific cellular effects are initially determined by a ligand-dependent or -independent receptor activation. Essential processes as cell growth and proliferation, cell differentiation, and apoptotic cell death can be initiated by this receptor.

This review gives an overview of the role of HER4 in normal and malignant breast epithelial cells and tissues. Specific mechanism of HER4 activation and subsequent intracellular signaling will be described by taking a focus on effects provoked by receptor shedding. HER4 activities and specific effects will be correlated to breast cancer subtypes and the impact of HER4 on course and outcome of disease will be considered. Moreover, current and potential therapeutic approaches will be discussed.

1. Introduction

1.1. A (very) brief history of the human epidermal growth factor receptor family

It was the Nobel Prize laureate of 1986 Stanley Cohen who reported already in 1965 on a specific, 53 amino acids consisting peptide, isolated from the submaxillary gland of mice that showed a stimulating effect on epidermal proliferation and keratinization [1]. According to its activity, which postulated a corresponding receptor protein, the proper noun Epidermal Growth Factor (EGF) was coined. Eighteen years later, in 1983, the molecular processing and secretion of EGF has been described by Axel Ullrich and collaborators [2] but soon after, the same research

group published in cooperation the successful cloning of the corresponding EGF-receptor gene (EGFR). The homology to v-erbB, the tumor inducing gene avian erythroblastosis virus became apparent and the oncogenic impact of EGFR gene amplification in epidermoid carcinoma cells has been defined [3,4].

It was again the group around Ullrich which (incidentally) cloned another gene with high homology to the EGFR gene and likewise with strong oncogenic capability. They called it Human Epidermal Growth Factor Receptor Related 2 gene (HER2/neu due to its discovery in neuro/glioblastoma; nowadays, the term “HER2” or alternatively “c-erbB2” is more common) [5]. A 2–50 fold HER2 gene amplification in about 30% of all breast cancers (BCs) that typically correlates with HER2 overexpression has been found soon after [6]. This discovery was an

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early milestone for the sub-differentiation of BC but also for the advent of one of the first antibody based target specific tumor treatments: By subsequent studies it turned out, again under the aegis of A. Ullrich, that a mouse derived anti-HER2 antibody (the so called clone 4D5) retarded the tumor growth of HER2 overexpressing BC xenotransplants in an appropriate mouse model [7,8]. 4D5 has been humanized by Genentech Inc., has been baptized trastuzumab (trade name Herceptin™) and could thereby be transferred to clinical applications [9]. Until today, the results of a clinical phase III trial published by Dennis J. Slamon in 2001 signify the basis for an anti-HER2 targeting with trastuzumab in combination with chemotherapy [10]. Since then the anti-HER2 targeting has been multifariously improved, extended, and combined with additional treatment approaches, for example the use of antibody drug conjugates, the application of a dual anti-HER2 targeting, the additional of administration of immunotherapies, and the use of modified trastuzumab administration. Thus, trastuzumab represented one of the first antibody based target-specific medications against cancer, which has been developed based on the model of oncogene driven carcinogenesis introduced by Bishop and Varmus [11,12]. Until today, the HER2 receptor represents the most relevant RTK in BC. It serves as prognostic marker and therapeutic target in so called HER2-enriched BCs for about 25 five years since the approval of trastuzumab treatment in 1998 by the Food and Drug Administration (FDA). Even though the anti-HER2 targeting has brought a phenomenal benefit for HER2-positive BC patients it is also accompanied by a significant rate of insufficient treatment response and even resistance.

The discovery of additional EGFR-related receptors proceeded basically by the research done by Gregory D. Plowman in the early nineties. He reported the cloning and expression of another EGFR-related receptor HER3 in 1990 [13] and in 1993 of a forth family member called HER4 [14]. By Plowman's research HER3/HER4 receptor specific ligands with receptor stimulating activity, so called Heregulins (in accordance to the receptor nomenclature) were also identified. Thus, in 1993 the four members comprising EGFR-family was complete - for the time being. In later years, it turned out that potentially more EGFR related receptor genes and protein-variants exist (see below). However, regardless of additional receptor variants, which evolved on the transcriptional level, the original receptor genes arose (in particular in mammals) by gene diversification during evolution. Accordingly, all HER receptors share a high degree of homology, both on the gene and the protein level. The nowadays commonly used nomenclature of the four receptor family members are EGFR or erbB1 and accordingly erbB2/3/4 receptors, or HER1/2/3/4.

Although RTKs of the EGFR family are commonly associated with an oncogenic activity their individual impact on the cellular behavior differs in some respects considerably, both in normal and malignant cells. This applies in particular to the HER4 receptor to which under certain circumstances tumor-suppressive activity has been attributed, a phenomenon, which can potentially be therapeutically exploited (see paragraph 2.7).

1.2. The principle of signal transmission via the epidermal growth factor receptor family

The HER receptors constitute a family of related receptor tyrosine kinases (RTKs), which show, due to their high degree of structural relationship, similar activity - at least to some extent. Accordingly, the receptor proteins are located in the outer cell membrane of normal or malignant cells, predominantly of epithelial (but also of neuroectodermal and mesenchymal) origin. In simplified form, typical receptor components consist of an extracellular ligand binding domain, a transmembrane domain, and an intracellular protein kinase domain. This molecular structure enables the transmission of signals from other cells and the cell environment across the cell membrane into the recipient cell. More specifically, the HER receptors combine the capacity of ligand (growth factor) binding that causes three dimensional,

conformational changes of the receptor itself [15] and, least as importantly, it triggers the tyrosine kinase activity that involves receptor cross- or auto-phosphorylation as well as the activation of downstream signaling molecules and pathways. However, the extracellular domains also come with molecular features that enable ligand-independent association that results in receptor activation [16–18]. The degree of self-association seems to be to some extent dependent on receptor density expressed on the cells [19]. Beside the general characteristics of EGFR members it is important to note that during evolutionary receptor diversification the HER3 receptor became kinase defective [20] while the HER2 receptor has lost the ability to bind any native growth factor, which is due to a tethered and thus closed conformation of the ligand binding domain [21–23]. Nevertheless, both receptor types are integral part of the functional receptor unit and even play a dominant role in the general signal transmission across the cell membrane. Both, HER2 and HER3 show pronounced oncogenic potential.

In normal tissues the HER receptors play a pivotal role for tissue and organ development, differentiation, and maintenance. During development the expression of all HER receptors is spatiotemporally strongly regulated and highly coordinated. In contrast, the absolute expression of specific HER receptors and coexpression patterns are frequently altered in malignant cells and tissues [24]. In normal tissues, especially the HER4 receptor expression and receptor-specific activity represent essential parameters for a well-coordinated and proper differentiation process (for more details see paragraph 2.3).

Numerous native ligands have been identified with individual binding specificity and affinity to individual HER receptors or receptor subsets, except HER2 (see above). Some of them dock exclusively to HER1, others to HER1 and 4, and still others to HER3 and HER4. A few have been described to be HER3- or HER4-specific. Thus, ligand-induced effects are also determined by individual HER receptor types expressed on target cells and the other way around: Different ligands which bind to the same receptor can trigger a different intracellular signaling machinery and can thus result in different cellular responses. Overall, the complex system consisting of (at least four) HER receptors and corresponding ligands has pleiotropic capacity. An overview of known HER receptors and corresponding ligands is given in Fig. 1.

Beyond individual and specific HER receptor activities the coexpression pattern is highly relevant for cellular effects and behaviors initiated upon ligand binding. Generally, the HER receptors do not autonomously trigger intracellular downstream effects but rather act in concert and as a functional unit. Ligand binding results in lateral receptor communication arranged by receptor interaction and cross-activation (i.e., typically cross-phosphorylation). Thereby, not only receptor homo- and heterodimers or oligomers can be assembled but on a larger scale also homo- and heteromeric receptor clusters can be formed [25,26]. (However, besides ligand-induced receptor interaction, an enhanced receptor overexpression can result in receptor assembly, as it has for example been described for the HER2 receptor; see above). The collective structural and enzymatic actions taking place at the membrane level upon ligand binding to the HER receptors determine the subsequent, intracellular events and finally the cellular outcome. This principle ensures not only the functionality but also the robustness of the whole HER receptor system. Consequently, slight, disruptive impacts on this system are normally well tolerated, however, substantial disturbances as receptor overexpression, an abnormal coexpression, hyperactivity (e.g., caused by receptor gene mutations), or the loss of receptor function result in irregular cell and tissue functions and potentially even in malignant cell transformation.

In reminiscence to a countless cited overview given by Yosef Yarden and Mark Sliwkowsky about two decades ago [27] the functional HER receptor unit consists of components, which can be attributed to a signal input layer (ligands), a signal transformation layer (RTKs), and a signal processing layer (intracellular pathways). Accordingly, the cellular responses, which are determined by this systems are subject of the output layer and include processes as cell growth and proliferation,

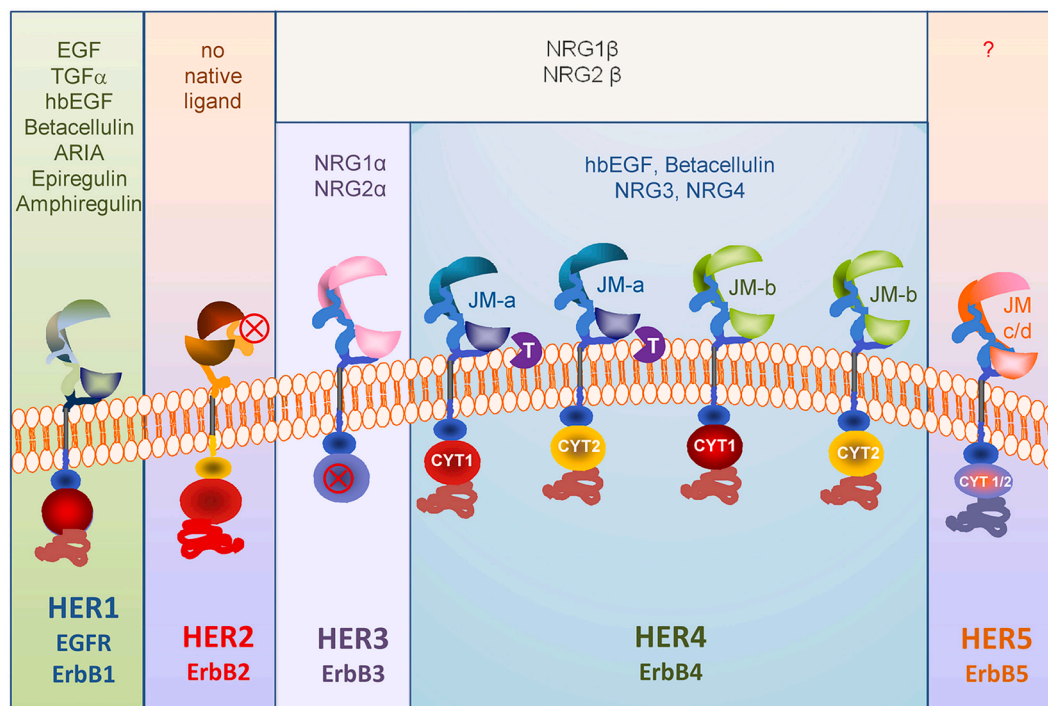


Fig. 1. Overview of the EGFR/HER1/erbB1-receptor family and corresponding ligands. From the evolutionary perspective the EGFR represent the progenitor from which by gene multiplication and diversification on the genomic level and by alternative splicing on the transcriptional level at least seven relatives originated from. The nomenclature “c-erbB” refers to viral respectively to the cellular *erythroblastic leukemia viral oncogene homologue gene* and “HER” to the *Humane Epidermal-Growth-Factor-Receptor Related* protein. All receptors belong to the class of receptor-tyrosine-kinases with a (potential) extracellular ligand binding domain, a transmembrane domain, and an intracellular kinase domain. A number of ligands bind to individual receptor subsets with either unique or multiple binding specificity, as indicated. Despite equal or similar binding specificity individual ligand can trigger different intracellular signaling pathways and thus can provoke different cellular behavior. During evolution, the HER2 receptor has lost the ability to bind any native ligand, which is due to a tethered, i.e., closed extracellular peptide conformation. Likewise, during receptor diversification the HER3 receptor became kinase defective. (These two special features are indicated by the red “cross-in-circle” symbol.) At least four different HER4 isoforms are known, with either an inherent extracellular JM-a or JM-b variant and alternatively an intracellular CYT1 or CYT2 domain. Only the JM-a version can be cleaved by TACE (i.e. the *disintegrin and metalloprotease* ADAM17) as indicated with the lilac symbol annotated with “T”. Moreover, additional HER receptor variants expressing extracellular JM-c or JM-d version have been identified and can be considered as additional HER4 isoforms or as HER5. However their potential role in BC (and other tissues and malignancies) is largely unclear, so far. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

For more details and references see main text. Abbreviation ARIA = acetyl-choline receptor inducing activity; TGFα = transforming growth factor alpha. hbEGF = heparin-binding epidermal growth factor. Other abbreviations are explained in the main text.

differentiation, adhesion and migration, and last but not least cell survival and death.

2. Molecular features of HER4, which make the difference

2.1. HER4 receptor phosphorylation

Overall, HER4-mediated signaling takes place at different levels and mainly involves receptor phosphorylation, potentially at multiple tyrosine residues, as well as (ligand-dependent and -independent) receptor cleavage, which causes release of (activated, i.e., phosphorylated) 4ICD into inner cell compartments (see paragraph 2.2). This process enables the interaction of the soluble 4ICD with a variety of downstream effector and target molecules.

The recruitment of specific signaling adapter and transducer molecules is in turn essentially determined by the phosphorylation of individual tyrosine residues at the internal receptor domain, a few of them within, but most of them beyond the kinase domain. Many of them have been found to enable docking of a different number of signaling molecules of specific pathways, which are known to (co-)regulate cell proliferation, survival, differentiation, and migration. Key pathways triggered upon phosphorylation of HER receptors are the pro-proliferative MAPK-pathway, the pro-proliferative and pro-survival PI3K/Akt pathway, pathways that involve PLCγ/PKC, but also those

that include janus kinases (JAKs) and signal transducers and activators of transcription (STAT) [28,29]. At least 18 tyrosine residues of HER4 (15 of them outside the kinase domain) have been identified to be get potentially phosphorylated by receptor activation and all of them have been attributed to trigger or to recruit at least one, most of them more adaptor or signaling molecules as well as corresponding pathways [30,31]. In addition, two different intracellular domains of HER4 show different preferences in recruiting downstream molecules and thus augment the signaling diversity (see paragraph 2.2 for more details).

A quite sophisticated approach has been applied by researchers affiliated to the Lab of Gavin MacBeath years ago [32]. The researchers used protein microarrays to decipher the complexity and promiscuity of signaling activity of all HER receptors (i.e., including HER4) on the level of single peptides. The specifically designed protein microarrays comprised virtually every Src homology 2 (SH2, $n = 109$) and phosphotyrosine binding (PTB, $n = 44$) domains encoded in the human genome and enabled the quantification of equilibrium/dissociation constants of each domain for 61 HER-related tyrosine peptides. Thereby, the researchers addressed specifically those molecules with the capacity to bind to tyrosine residues, exhibited by potential interaction partners. The approach allowed not only to identify receptor and tyrosine residue-specific molecule interaction but also to determine respective binding affinities and thus to quantify minimal protein concentrations, which are required to enable functional contacts. A main finding was that HER4

shows relatively sparse amount of protein connections (in contrast to e. g., HER2 that shows the greatest diversity of signaling by recruited proteins). It has been concluded that (despite the presence of a high number of potential phosphorylation sites) HER4 serves a more specialized function than the other HER receptors, however, missing sites of tyrosine phosphorylation could not safely be excluded in this study.

Three years later the same group extended the study on potential interactions of tyrosine phosphorylated HER4 with signaling molecules. Again they found, this time by tandem mass spectrometry, HER4 to be substantially more selective than the other HER4 receptors in recruiting just a small subset of those signaling molecules, which are known to interact with other HER receptors [33]. This is remarkable, because not less than nineteen potentially phosphorylated tyrosine residues at the intracellular HER4 receptor have been identified in the cited study. Nevertheless, the pronounced selectivity has been interpreted as a plausible explanation for its protective role in cancer cells (see below). More specifically, the presence of HER4 may decrease the oncogenic capacity of cognate HER receptors (in particular with HER2 and HER3) in favor of less malignant activities of heterodimeric receptor complexes.

2.2. HER4 receptor isoforms and receptor processing

The complexity of the EGFR family, which is due to receptor variants and numerous ligands with different specificity and activity is further increased by the generation of additional HER4 receptor isoforms generated by differential splicing. Those HER4 receptor variants have been identified and characterized in-depth by Klaus Elenius and co-workers [34–36]. Four basic HER4 versions have been described in detail and have been named JM-a/CYT1, JM-a/CYT2, JM-b/CYT1, and JM-b/CYT2 (while the abbreviation JM and CYT refer to the extracellular/juxtamembraneous and the cytoplasmic domain, respectively). Overall, the HER4 gene comprises 29 exons and the two extracellular domains differ in being encoded by exon 15 (JM-b) or exon 16 (JM-a) and by the presence (CYT1) or absence (CYT2) of exon 26 encoded mRNA [36,37].

Critical features of HER4 isoforms include that only the JM-a (but not the JM-b) variant can be cleaved by disintegrin and metalloprotease-17 (ADAM17), also known as the Tumor necrosis factor- α converting enzyme (TACE), which results in the shedding of an extracellular domain (4ECD) [38]. The extracellular, proteolytic processing occurs basically ligand-independently but seems to be enhanced by the impact of estrogen and HER4-specific ligands as Neuregulin-1 (NRG-1) [39–41]. Subsequently, the truncated HER4 can be subject of an intracellular cleavage by γ -secretase. Thereby, a soluble intracellular domain (4ICD) with signaling activity is released into inner cell compartments. Intracellular routing and signaling effects of 4ICD will be discussed further on. In general, the TACE and γ -secretase related two-step cleavage is not unique and does not exclusively act on HER4. Instead it is a known mechanism for the activation of membrane located receptor molecules and corresponding signaling pathways, amongst them a number of Ephrin (EPH) receptors, mesenchymal-epithelial transition (c-MET) receptor, some fibroblast growth factor receptors (FGFRs), insulin-related receptors (IRR), and last but not least Notch (incomplete list) [42,43]. Nevertheless, the release of 4ICD by γ -secretase has been first described by Ni et al. in 2001 [44]. In contrast, HER1, 2 and 3 receptors do not represent substrates of TACE and γ -secretase (although HER2 is also known to occur as a truncated version upon extracellular shedding by other proteases than TACE [45]).

Regardless of an HER4 two-step cleavage and the subsequent release of 4ICD into the cell interior the CYT1 and CYT2 domains show different signaling activity. It has been reported that the two intracellular receptor components interact with various adapter molecules and trigger different intracellular pathways with domain-specific priorities. For example, the pro-proliferative MAPK/ERK pathway has been described to become activated via SHC by both the CYT1 and CYT2 4ICDs, whereas

the pro-proliferative and pro-survival PI3K/Akt pathway is supposed to get mainly triggered by CYT1 but rather not by CYT2 [36]. However, it can be speculated that these differences are most probably less relevant because most cell types do not exclusively express either CYT1 or CYT2. This has been explicitly shown for BCs separated by the ER status [39]. Instead, it can be assumed that neither the CYT1 nor the CYT2 expression does develop a functional predominance. Nevertheless, the general ability of the 4ICD to provoke either pro-proliferative and pro-survival or pro-apoptotic activity (in particular upon intracellular cleavage and release) has been to some extent associated to isoform specificity. The cytoplasmic retention (rather than the translocation to the cell nucleus) seems to be preferably achieved by the CYT1 isoform [46]. However, cytoplasmatically expressed molecules with steering function for 4ICD within intracellular compartments probably have much greater impact on the cell's fate, an issue that is not only but highly relevant in malignant cells (see paragraph 2.4 and 2.6.2).

2.3. Impact of HER4 on the mammary gland development and differentiation

The HER4 receptor has been found not only to be involved in but also being essential for the development of some organs and tissues as the heart, central nervous system, kidney, salivary gland, testis, mammary gland, and others more [37]. However, HER4 expression density and the occurrence of individual isoforms vary and seem to be tissue specific. This also implies that different cell and tissue types express cleavable (JM-a) or non-cleavable (JM-b) HER4 isoform and those with either CYT1 and CYT2 cytoplasmic domains.

The importance of HER4 for the differentiation and regular function of mammary gland is being known for many years. Already in 1999, Frank E. Jones inactivated HER4 in the mammary gland of a transgene mouse model by introducing a HER4 dominant-negative allele [47]. He observed the lack of normal luminal lactation products as α -lactalbumin and thus an insufficient mammary terminal differentiation. In addition, the HER4 signaling that drives the differentiation during mouse mammary gland development could be attributed to a proper timing of HER4 activation during this process. A regular and pronounced HER4 tyrosine phosphorylation was observed especially at 14 days postpartum during mid-lactation [47].

On the molecular level the deficient differentiation could be attributed to an inactive Signal Transducer and Activator of Transcription 5A (STAT5A). The importance of STAT5A for the differentiation of mouse mammary epithelial cells during pregnancy and of human mammary epithelial cells in-vitro has been highlighted already 30 years ago [48–50], however the direct link between HER4 and STAT5A has been discovered years later [51]. In particular, the cleavage mediated generation of the HER4-related 4ICD has been suggested to be a critical step for the activation of STAT5A by HER4 and thus for lactogenic differentiation [52–54]. This mechanism has been specified by Han et al. (Frank Jones group), who described a detailed molecular mode of action that explains the direct HER4/STAT5A interaction, which essentially contributes to mammary epithelial cell differentiation [55]. Han et al. provided evidence for a direct coupling of HER4 and STAT5 via a functional STAT5-SH2 domain that requires phosphorylation at Y984, which is impaired or completely absent in HER4 deficient mouse mammary cells. Moreover, Han observed that a 4ICD promoted differentiation process in HC11 mammary epithelial cells is related to significantly increased expression of STAT5A target genes and the differentiation markers β -casein and the so called whey acidic protein (WAP). In contrast, cells stably expressing 4ICD with a molecular replacement of the tyrosine Y984 by an alanine F984 failed to undergo differentiation. The corresponding molecular model suggests that only an intact 4ICD/STAT5A molecule complex enables the gene transcription of differentiation markers in mammary epithelial cells, which is finally accomplished by the transcriptional activity of STAT5A itself. Insofar, 4ICD acts as chaperone for STAT5A [56] (but for other

transcription factors as well).

Importantly, the complete mechanism involves the above-mentioned and preceding HER4 receptor shedding by TACE and γ -secretase and thus the intracellular release of 4ICD. Accordingly, this signaling activity requires the expression of cleavable JM-a isoforms. Moreover, the initial HER4 receptor cleavage is enhanced by the impact of HER4 ligands as Heregulin (HRG), which are, according to the aforementioned effects, also referred to as Neu Differentiation Factors (NDF) or NRG [57–59]. Independently of a direct interaction of 4ICD and STAT5A a prolactin and Janus Kinase 2- (JAK2) dependent STAT5A activation has also been identified [60]. Basically, a simultaneous activation of the prolactin receptor and HER4 obviously enhances STAT5A transcription activity, which is definitely a hallmark of lactogenic differentiation. Within this molecular interplay also JAK2 represents a critical mediator for HER4 mediated differentiation.

Interestingly, not only HER4-specific ligands as HRG but also the exposition of BC cells to estrogen enhances the HER4 ectodomain shedding [61]. The potential clinical importance for the diagnosis and treatment of BC will be discussed further down. Nevertheless, this phenomenon implies that various external (soluble) factors, as components of the environment, either HER4-specific or not, induce or at least affect the molecular state of HER4 and its activity.

Also noteworthy is that sequential processes of a regular mammary gland development are differentially affected and controlled by HER4 receptor molecules, which metachronously exhibit either CYT1 or CYT2. Wali et al. correlated HER4-CYT1 vs. HER4-CYT2 receptors with various developmental stages of the glands in a mouse mammary tumor virus (MMTV) based HER4 transgenic mouse model [62]. He demonstrated that the mammary ductal growth and branching in eight week old “HER4 CYT1 mice” was significantly diminished compared to non-transgenic sibling controls or compared to “HER4-CYT2 mice”. This observation could be verified in elder and pregnant mice but not in the same mice 16 days post-partum, which suggests an alternating HER4 isoform expression at respective maturation states. However, in this particular mouse model a correlation of intracellular pathways specifically but differentially triggered by HER4/CYT1 and HER4/CYT2 has not been shown. Nevertheless, HER4 could unambiguously be attributed to pro-differentiation phenotypes, which is rather manifest late in pregnancy and during lactation, while a differential expression of CYT1/CYT2 HER4 isoforms has been documented during puberty and pregnancy.

2.4. Prognostic and predictive impact of HER4 and its isoforms in breast cancer

Over the past years, somewhat inconsistent data circulated with respect to the importance of HER4 in BC. A series of analyses attributed an unfavorable impact of HER4 expression on the course of disease, while others revealed positive effects.

One of the first reports that HER4 might favorably affect BC disease was published by Caroline Witton, who evaluated the expression of HER1–4 on course of BC disease simply by immunohistochemistry but basically regardless of BC subtypes [63]. A differential analysis accomplished within this study, revealed, however, that a (pronounced) HER4 expression in BC was associated with more differentiated tumors and with a favorable impact in particular within the ER-positive BC sub-cohort. By a follow-up study the same group provided evidence for anti-proliferative activity of HER4 in primary BC [64]. Taken together, both analyses suggested that, due to the positive impact of HER4 on the outcome of (specific) BC diseases, a therapeutic pan HER receptor targeting (e.g., in case of an insufficient response to an anti-HER2 treatment) might not categorically be advised, at least not for HER4-positive tumors. It should be noted that, in contrast to Witton's study, in the same year another immunohistochemical analysis was applied, even though to a relatively small ($n = 66$) sample collective of node positive stage II–IIIa BCs, that revealed an adverse prognostic impact of pronounced

HER4 expression on the outcome of disease [65].

Short time later, in 2005, evidence has been provided for HER4 to favorably affect the risk of recurrence of ductal breast carcinomas in-situ [66]. Barnes et al. analyzed the occurrence of HER2, HER3, and HER4 in local and invasive BC and found HER4 to be associated with significantly longer periods of relapse-free disease. In the same study it has been demonstrated that the presence of HER4 reduces the risk of recurrence both in HER2-negative and HER2-positive BCs (while the HER3 receptor had the opposite effect). However, it must be noted that these analyses included BCs originated from different taxonomic subgroups (i.e., ER-positive, HER2-positive etc.), thus, a potentially different impact of HER4 in specific subtypes has not been elucidated. Nevertheless, a longer relapse-free survival and thus a better prognosis for high-risk early BC patients with pronounced levels of tumor-associated HER4 mRNA was not much later found by Koutras et al. who generally confirmed the favorable impact of this receptor type [67,68].

2.5. HER4 in HER2-positive and triple-negative BC

In 2008, our group analyzed the impact of enhanced HER1–4 gene dosages on the outcome of 278 invasive breast cancer diseases [69]. In compliance with existing data we found that the HER2 gene locus showed the most pronounced gains, however also HER1, HER3, and HER4 gains were found, even though to a less degree. Interestingly, we could demonstrate that a gain of the HER4 gene correlates with a better outcome of disease, an effect that was even more pronounced in BC patients who suffered from HER2-amplified tumors compared to those, who had tumors without an HER2 gain. At that time, these data suggested some kind of HER2/HER4 or HER4/HER4 interaction, which might have reduced the malignant manifestation of HER2 in those tumors (see also the recent study by Miano et al. further down [70]).

Soon after, we specifically analyzed the impact of HER4 in HER2-positive BC patients who received trastuzumab therapy compared to those, who were not subjected to a HER2-specific treatment [71]. We demonstrated that patients with HER2/HER4 double-positive tumors had a significant better cumulative survival than those with HER2-positive/HER4-negative tumors. This effect was even more pronounced in the trastuzumab-treated cohort, which was indicative for a better treatment response when tumor cells showed enhanced HER2 expression and a relevant amount of HER4. In terms of intracellular signaling higher p27^{Kip1} expression values and higher HER2 phosphorylation rates also correlated with an improved therapy response. The finding of an improved response to trastuzumab treatment of HER4 positive tumors was later confirmed by others [72]. More specifically, Portier et al. revealed HER4 with a significant predictive ability in identifying patients with improved metastasis free, progression free and overall survival (OS). The favorably impact of HER4 came into effect both in early HER2-positive and advanced, i.e., metastatic BC. In accordance with the aforementioned reports a very recent study based on mRNA expression analysis again revealed a prolonged relapse-free survival of patients suffering from HER2-enriched (and luminal A) BC disease [70].

However, evidence has been raised that the subcellular localization of HER4/4ICD can make a difference (not only in ER-positive as outlined below, but also) in HER2-positive BC cells with respect to the response to trastuzumab treatment. Mohd Nafi observed in Heregulin and trastuzumab treated HER2-positive BC cells a pronounced nuclear HER4 translocation [73]. In contrast, the experimental inhibition of HER4 cleavage by a γ -secretase inhibitor resulted in enhanced trastuzumab sensitivity. Those data substantiated the finding that course and outcome of HER2-positive trastuzumab treated patients was significantly worse in case of a pronounced detectability of nucleus-located HER4 compared to those patients with tumors that showed preferentially extra-nuclear HER4. Thus, the study by Mohd Nafi provided evidence that HER4 not only as a whole cell-membrane located receptor molecule can impair the trastuzumab treatment efficiency (for instance

via specific receptor interaction, or by compensating signaling from the cell surface) but in particular as a cleaved HER4 subunit with nuclear localization. However, a specific, HER4-related intra-nuclear mechanism in the HER2-positive BC subtype has not been suggested.

By a follow-up study of our aforementioned investigation, we drew our attention to the impact of HER4 in both, HER2-positive and triple negative BC (the latter clinically negative for HER2, ER, and the progesterone receptor PR), this time taking HER4 isoforms into consideration [74]. To this end, we applied a quantitative PCR analysis of transcriptional HER4 expression based on an molecular approach published by Junttila et al. [39]. In accordance with the results reported therein a not-cleavable HER4 receptor isoform (i.e., juxtamembraneous JM-b variants) were not detectable in any of the analyzed samples. However, the coexpression ratios defined by JM-a/CYT1 vs. JM-a/CYT2 variants varied considerably. Notably, we found a significant longer cumulative event free survival (EFS) of patients with HER2-positive cancer and a longer OS of TNBC patients, when the HER4 expression on tumor cells exceeded a critical value. However, high vs. low HER4-CYT1/HER4-CYT2 isoform expression ratios did not cause any distinct or disparate effect on the course of disease, although numerous CYT1 and CYT2 specific pathways and transcriptional regulators have been predicted [75]. It should be noted that our study did not differentiate between potential subcellular HER4/4ICD locations, which might have special relevance (see 2.6.1).

In contrast to a number of previously generated data a study performed by Kim et al. revealed a reduced relapse free survival (RFS) of TNBC patients with higher HER4 expression values (determined by NanoString technology) compared to those with low HER4 expression [76] whereas the impact of HER4 on the OS was not analyzed. Overall, the predictive impact of HER4 in HER2-positive (and triple-negative) BC remains somewhat uncertain and requires a deeper mechanistic exploration of HER4 expression, its isoforms, its cleavage products, and its subcellular activity in these BC subtypes.

2.6. HER4 in ER-positive (i.e., luminal) BC

Overall, there is accumulated evidence for a favorable prognostic impact of HER4 in luminal BC. Based on clinico-pathological well defined tumor cohorts it has been shown, for example, that a low HER4 expression is associated with pronounced tumor progression, while patients with ER/HER4 double-positive (in contrast to ER-positive/HER4-negative) tumor show an improved prognosis [39,63,65,72,77]. (This is not necessarily in contradiction with an useful and successful therapeutic anti-HER4 targeting of luminal BC under certain conditions; see paragraph 2.7). The favorable impact of HER4 expression has been attributed to pro-apoptotic rather than oncogenic activity of HER4. Experimentally, Das et al. (Frank Jones' group) could elicit this tumor cell destructive activity of HER4 by reactivation of epigenetically silenced HER4 expression [78].

Elenius' research group from the University of Turku reported in 2005 that, compared to non-malignant breast epithelium, ER-negative BCs expressed a slightly, and ER-positive BCs a considerably elevated amount of HER4 [39]. Data recently provided by Miano et al. also suggest highest HER4 mRNA levels in luminal BC and lowest in basal-like TNBC samples, while intermediate levels were found in HER2-enriched tumor tissues [70]. These findings strongly indicate that HER4 expression might have a major impact in luminal BC compared to other BC entities, which in turn suggests an influence of HER4 on ER-dependent tumor growth. As mentioned above, another relevant finding was, unlike in normal breast tissues, the expression of JM-a but never JM-b isoforms in BC tissues [79]. This observation implied a potential cleavage-dependent (and potentially ligand independent) HER4 receptor activation in malignant BC cells. However, not only HER4-specific ligands but also the impact of the steroid hormone estrogen stimulates the HER4 ectodomain shedding [61]. Accordingly, higher concentrations of shed HER4 ectodomain have been identified in the

serum of patients suffering from hormone-dependent BC than in comparative samples taken from ER-negative BC patients. In addition, elevated serum ectodomain concentrations were associated with the premenopausal status at diagnosis. These and numerous more evidences suggest that HER4 has a greater impact in ER-positive than in other BC subtypes. Nonetheless, reports on the prognostic impact of HER4 in luminal BC are partially inconsistent (see also below). Even if HER4 receptor shedding suggests its activation, both a stimulating and inhibiting effect on tumor cell growth and thus a favorable and an unfavorable impact of HER4 expression and activity has been reported for the luminal BC subtype. An example for an improved course of estrogen receptor-positive BC disease in terms of a prolonged relapse-free survival is given by the very recent report given by Miano et al. [70]. Interestingly, HER4 had this effect only in luminal A cancers whereas in luminal B samples no effect of HER4 was seen. Evidently, the HER4 activity depends on numerous molecular parameters, as discussed hereinafter.

2.6.1. Intracellular activities of HER4 in luminal BC – The subcellular localization matters

Various reports on the prognostic value of HER4 in BC were somewhat inconsistent and apparently contradictory. Other studies revealed that the prognostic impact of HER4 does not only depend on its expression status but mainly depends on the subcellular localization of HER4 (or rather of 4ICD). However, the findings were again conflicting.

In 2009, it was again F. Jones and his team who reported the first time that cytosolic (instead of nuclear) localization of the cleaved HER4 (i.e., 4ICD) has a significant positive prognostic value in BC patients with invasively growing BC ($n = 923$). The multivariate data analysis held in particular true for the cohort classified as lymph node-negative [80], however, a specific discrimination of ER-positive vs. ER-negative tumor patients was not made. Nonetheless, Jones and coworkers could verifiably refer to previously in-house generated data, by which Naresh et al. experimentally demonstrated the capacity of cytosol-located 4ICD (rather than membrane-associated HER4) as a “BH3-only” protein (see below) for mitochondrial accumulation. Here, 4ICD directly interacts with the “apoptosis regulator protein” BAK with pro-apoptotic activity [81]. The in-vitro experiments further revealed that at the same time the activity of anti-apoptotic molecules is reduced by 4ICD. Overall, cytosolic 4ICD is capable to trigger a cascade of canonical apoptotic pathways, which involve mitochondrial membrane permeabilization, subsequent cytochrome-c efflux, and finally the activation of cysteine proteases, referred to as caspases with caspase-3 as key-player. As a result, the cells underlie intrinsically initiated apoptotic cell death, a mechanism that explains the role of HER4/4ICD as a favorable prognosticator, but only when located in the cytoplasm rather than in the cell nucleus. Interestingly, such a HER4/4ICD mediated apoptosis seems to be Heregulin-dependent and requires subsequent HER4 cleavage. However the 4ICD-mediated pro-apoptotic effect has been even found in the absence of kinase activity, which justifies the general conclusion that HER4/4ICD triggered intracellular signaling occurs in part kinase-dependently and -independently. Due to the kinase-independent trigger of cell apoptosis 4ICD has been identified as a member of the so called molecule with Bcl-2 homology class 3 (i.e., a “BH3-only protein”) and thus has been attributed to the group of pro-apoptotic Bcl-2 molecules [81]. Molecular mechanisms, which enable and facilitate a cytoplasmic localization and pro-apoptotic activity of 4ICD will be addressed further down.

By a subsequent study the same research group put the action of HER4/4ICD into the context of a tamoxifen (TAM) based endocrine treatment (ET) of estrogen receptor-positive MCF-7 and T-47D BC cells in-vitro and in a mouse based xenograft model [82]. Thereby they could show that HER4 mediates TAM-induced cellular apoptosis of ER-positive BC cells and suggested a mechanism by which TAM disrupts a molecule complex consisting of the ER and 4ICD. In addition, the TAM induced ER/4ICD disruption entailed 4ICD mitochondrial accumulation, which

again associated with apoptotic cell death. The 4ICD-BH3-only part was essential in regulation of the TAM activity in-vitro and in the preclinical in-vivo model. Moreover, the analysis of tissues derived from TAM-treated patients indicated that mainly tumors with nuclear 4ICD were responsive, however, the sample number included into this analysis was very small. Nevertheless, this and similar studies provided strong evidence that not only the presence or absence of HER4 has a prognostic impact in (luminal) BC, but also HER4 cleavage and the subcellular localization of 4ICD affect the (endocrine) treatment efficiency and thus might have a predictive value.

We substantiated the observation that the HER4 receptor affects the TAM treatment efficiency in-vitro. We have shown that ER/HER4 double positive ZR-75-1 BC cells were basically sensitive to TAM treatment, however, the treatment efficiency, represented by a reduced cell cycle progress and an increased fraction of quiescent (i.e., not cycling) cells could be considerably enhanced by a HER4 receptor knock-down [83]. Based on additional functional studies, by which 4ICD has been identified to operate as ER-coactivator [82,84,85], we assumed that a nuclear 4ICD and ER complex / interaction impairs the inhibitory effect of TAM, while the HER4 knock-down disables this interaction and thus raises the treatment efficiency [86]. We complemented the experimental evidence by quantitative analysis of HER4 expression in 258 BC specimens and identified a poor course and outcome of premenopausal and TAM treated BC patients with HER4-positive tumor tissues, whereas TAM-treated patients with low or absent tumor-associated HER4 expression performed significantly better. Notably, the fact that no (unfavorable) effect of HER4 could be deciphered for patients treated with an aromatase inhibitor (AI) supported the trilateral ER-4ICD-TAM interaction.

However, also for luminal BC (cells) some conflicting data are circulating. For example, Göthlin-Eremo et al. could not identify an independent predictive significance of HER4 with regard of TAM treatment [87]. Furthermore, Fujiwara et al. could correlate a pronounced nuclear occurrence of 4ICD with a favorable effect, especially in patients with the ER-positive/HER2-negative BC treated with endocrine therapy [79]. Moreover, the group associated this observation with a CYT-2 dominance of nuclear 4ICD and the absence of cytoplasmic 4ICD consisting of CYT-1 and speculated that a nuclear 4ICD indicates its main site of action as ER-coactivator (which is basically in agreement with above mentioned data). However, that explicitly the coactivation of the ER via 4ICD should represent a mechanisms involved in better prognosis is somewhat questionable (see below). At least, the finding is hardly compatible with a pro-proliferative effect mediated by some kind of 4ICD/ER interaction. Instead, an ER-coactivation that promotes cell differentiation rather than proliferation might be conceivable. However, such an effect has been rather attributed to an 4ICD/STAT5A interaction and STAT5A-related transcriptional activity (see above).

2.6.2. HER4 activation and subsequent intracellular routing of 4ICD in (luminal) BC

As pointed out above, the effect of activated or shed HER4 is manifold both in normal and malignant breast epithelium and involves a regulating impact on cell proliferation, differentiation, survival, and death. Consequences of specific HER4 signaling become obvious by considering the different intracellular routing of the (activated) 4ICD, which entails fundamentally different and even opposite cellular responses. Accordingly, HER4 cannot be considered per se to either act as an oncoprotein or a tumor-suppressor. Indeed, the HER4 related mode of action depends on a variety of factors, which can be attributed to three levels of receptor function: 1.) Way of receptor activation, i.e., ligand-dependent vs. ligand-independent that results in receptor phosphorylation and the activation of signaling cascades or/and receptor shedding followed by the release of 4ICD into inner cell compartments. 2.) In terms of ligand-dependent HER4 activation a considerable diversity of ligands enables various cell responses (Fig. 1). Amongst all HER4 binding ligands NRG3 and NRG4 have been shown to exclusively dock to HER4 while NRG1 β and NRG2 β show less receptor specificity and bind

not only to HER4 but also to HER3. The HER4 ligand diversity is even increased by derivatives of the Epidermal Growth Factor (EGF) namely heparin binding EGF Like Growth Factor (hbEGF), epiregulin, and betacellulin. Due to a their dual binding specificity the latter three growth factors trigger HER4 but also the EGFR [28,88,89]. Since the ligands listed here dock to unique extracellular HER4 epitopes their binding consequently triggers different and cell-specific effects. 3.) A third level of HER4-specific activities is determined by the cell-specific intracellular molecular context. More specifically, the interaction of phosphorylated tyrosine residues with adaptor and signaling molecules depends on their availability to interact with the 4ICD. In addition, the intracellular routing of the 4ICD, i.e., the preferred cytoplasmic, mitochondrial, or nuclear localization is mainly directed by additional molecules expressed in inner cell compartments.

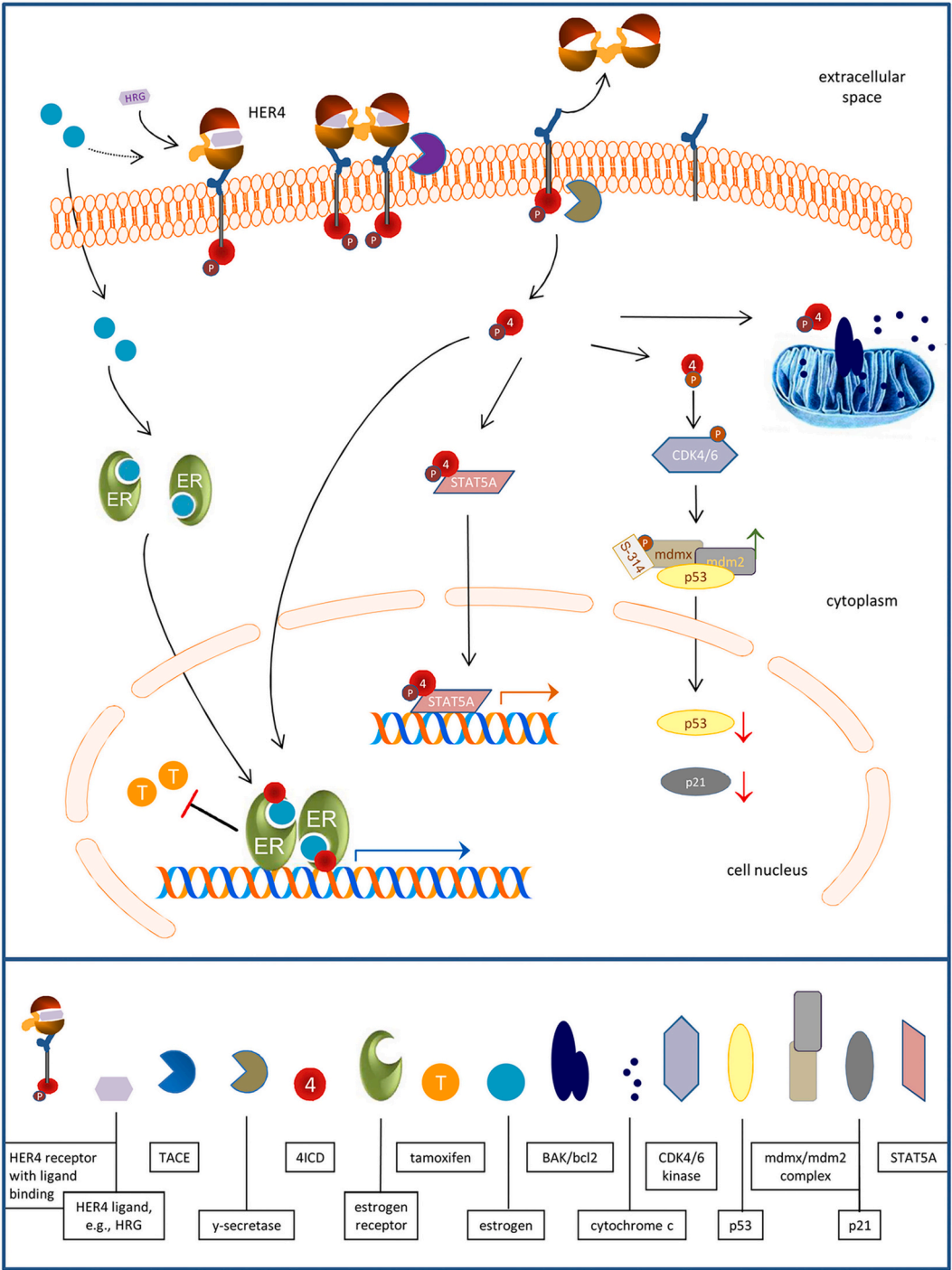
The differential routing of 4ICD is particularly relevant in ER-positive BC, because the effects caused by 4ICD largely depend on its intracellular location. Upon HER4 cleavage the released 4ICD can translocate into the nucleus and potentially activates (or suppresses) a variety of transcription factors, amongst them the already above mentioned STAT5 [56] but also *yes-associated protein* (YAP) [90], the transcriptional corepressor *Eight-Twenty-One* ETO2 (also known as MTG16 or CBFA2T3) [91], the *TGF-Beta Activated Kinase 1* (MAP3K7) *Binding Protein 2* (TAB2) [92] (which also complexes with the corepressor NCoR), and last but not least with the ER. As already discussed, a number of data imply a pro-proliferating activity of 4ICD upon interaction with ER which drives malignant cell growth [39,85,93]. Therefore, it is no surprise that a pronounced nuclear localization of 4ICD has been repeatedly associated with a rather poor prognosis for luminal BC patients [80,84]. Independently from its prognostic impact the presence of the HER4 receptor and in particular its nuclear localization also unfavorably affects endocrine treatment efficiency in patient with ER-positive BC, thus the presence of HER4 and the location of 4ICD has predictive value for this specific BC subgroup [54,82,83,90].

In contrast to a nuclear localization, a preferred extra-nuclear i.e., cytoplasmic presence of 4ICD can be considered to have a beneficial impact on the course of (luminal) BC disease. As outlined above, this phenomenon can be explained by the fact that 4ICD comprises a BH3-only protein domain which enables the interaction with pro-apoptotic molecules (partially) located in the mitochondrial membrane. As a molecular key player the *Ww Domain Containing Oxidoreductase* (Wwox) prevents nuclear translocation of 4ICD [94,95]. Another actor that shifts the balance of 4ICD location towards extra-nuclear compartments is Itch, which as an E3 ubiquitin protein ligase also enables the intracellular degradation of 4ICD [96]. Thus, a tumor suppressing activity has been associated to Wwox and Itch and they indicate a rather improved prognosis of tumor diseases [97–99]. Importantly, the interaction of Wwox and Itch takes place preferably with CYT1 containing HER4 variants, which is due to the presence of an extra ww-binding motive of this isoform [46,79,99]. To what extent this molecular specificity between CYT1 or CYT2 containing HER4 receptors indeed plays a relevant role is questionable, because (as mentioned above) JM-a/CYT1 and JM-b/CYT2 isoforms have been found always simultaneously expressed in BC, even though at different ratios [39,74,79].

The differential intracellular routing and molecules involved in regulating the intracellular distribution of 4ICD are illustrated in Fig. 2.

2.7. The potential of therapeutic anti-HER4 targeting

Both, preclinical in-vitro and in-vivo analyses as well as retrospective evaluations conducted on primary BC cohorts generated accumulated evidences for a considerable predictive power of HER4, in particular in luminal BC. As outlined above, HER4 has been shown to directly (and indirectly) affect the TAM treatment efficiency [83]. If clinically and prospectively confirmed and substantiated that a relevant amount of HER4 adversely affects the endocrine treatment efficiency or that it enhances the probability for early relapse it might be useful to utilize



(caption on next page)

Fig. 2. The figure illustrates the four most relevant actions of 4ICD upon HER4 receptor shedding. The extracellular JM-a domain of HER4 can be cleaved by TACE, which causes initially the release of 4ECD into the environment. Subsequently, the proteolytic action of γ -secretase results in the release of 4ICD into the cell interior. This two-step HER4 processing has been shown to be enhanced in the presence of estrogen and the HER4 ligand HRG. Thus, HER4 phosphorylation and cleavage is not mutually exclusive but can take place hand in hand. Within the cell 4ICD can take different routes and can be found located in the cytoplasm, associated to the mitochondria, or located primarily in the cell nucleus. The action of 4ICD in certain subcellular compartments affects and regulates the balance between cell proliferation, cell differentiation, and cell survival/death.

1) Upon translocation into the nucleus 4ICD is supposed to interact with a number of transcription factors, amongst them the ER, which is highly relevant in luminal BC. Relevant transcriptional targets are known to drive cell proliferation. In addition, HER4 has been found to reduce the treatment efficiency of ER positive BC cells with TAM. It has been suggested that 4ICD as a coactivator directly interacts with the ER and thereby mediates an opposite effect than TAM. Alternatively, 4ICD might somehow simply compete with TAM for ER binding and thereby might prevent or at least reduce a TMA/ER interaction.

2) Alternatively, 4ICD can be hindered from a translocation into the nucleus (e.g., by *wwox*, YAP, and other molecules, for more details see text) so that 4ICD takes effects in extra-nuclear compartments. Upon receptor cleavage, 4ICD stabilized a molecular complex of mdmx and mdm2 by CDK4/6-dependent phosphorylation of mdmx at Ser314. An active mdm2 ubiquitin ligase facilitates p53 proteasomal degradation, which entails reduced p21 expression and finally results in a loss of cell cycle control (and together with a decreased presence of p53 in a reduced capacity for apoptotic cell death). Thus, this mechanism presents another way how HER4/4ICD potentially drives cell proliferation. (Alternatively, 4ICD itself can be directly subjected to proteasomal degradation within the cytoplasm, e.g., via Itch; not graphically illustrated).

3) When withheld in the cytoplasm, 4ICD has been shown to interact via its BH3 domain with pro-apoptotic molecules, e.g., BAK and bcl2, which are typically located in the mitochondrial membrane. An activation of these molecules drives apoptotic cell death via the release of cytochrome-c from mitochondrial compartments.

4) The cleaved HER4 also promotes the terminal cell differentiation of mammary epithelial cells via 4ICD. In particular, the Y984 phosphorylation of the HER4 enables a direct 4ICD/STAT5 interaction, which determines the transcription of Casein and WAP via STAT5 and thus enables cellular differentiation.

For more details and references see main text.

Overall, the 4ICD regulates, controls, and affects cell proliferation, differentiation, and death of mammary epithelial cells via direct molecule interaction (of the released 4ICD) within inner cell compartments. Shifting the action of 4ICD towards cell differentiation and cell death and preventing a pro-proliferating activity enables novel therapeutic intervention, especially for ER-positive BC.

HER4 as a therapeutic target itself. This can most specifically be done by HER4-targeted antibodies. First ideas to specifically target the HER4 receptor by an antibody treatment and thereby to mimic NDF-induced cell differentiation have been presented in the ninetieth, already [100]. This strategy has been picked up by others, not least by the Elenius' group a number of years ago. M. Holmén et al. presented a monoclonal, mouse-derived anti-HER4 antibody called Ab1479 with growth-inhibiting capacity of HER4-positive BC cells in-vitro [101,102]. Ab1479 was shown to dock with high specificity to the cleavable JM-a isoform of HER4 and thereby to prevent receptor cleavage and phosphorylation. In addition, HER4 downregulation and subsequent ubiquitination has been observed upon cell treatment with Ab1479, which entailed receptor degradation (probably via recruiting the ubiquitin ligase c-Cbl) as well as suppression of anchorage-dependent and -independent cell growth of e.g., ER-positive MCF-7 BC cells in-vitro. Those anti-tumorigenic effects might be even enhanced by antibody triggered immunological mechanisms such as Antibody Dependent Cell-mediated Cytotoxicity (ADCC). Nevertheless, applications of antibodies with potential therapeutic activity require further evaluation in preclinical in-vivo models and later on in the clinical setting. However, for advanced in-vivo studies Ab1479 would require chimerization or preferably humanization beforehand.

Other rat-derived anti-HER4 antibodies have been generated and functionally tested by Okazaki et al. [103]. One particular IgG, named "P6-1", reduces HRG1-dependent HER4 phosphorylation and downstream signaling in strongly HER4-positive T-47D cells. In addition, growth of MCF-7 in 3D matrigel-based cell culture was inhibited when exposed to "P6-1", which again showed HRG1/NGR1 competing effects on BC cancer cells. Since NRG1 has been shown to stimulate proliferation of (BC) cells anti-HER4 antibodies with NRG1-opposing activity might be inherently useful for therapeutic purposes.

The idea of an antibody based HER4 targeting has been continued by Lanotte et al. who - not so long ago - generated a number of monoclonal anti-HER4 antibodies via phage display technology [104]. Advantageously, those antibodies originate from humane species that enables potential future (pre-)clinical applications. Lanotte et al. analyzed in-vitro diverse molecular effects in HER4-positive (BC) cancer cells upon anti-HER4 treatment. In addition, he applied HER4 transfection to inherently HER4-negative cancer cells and exposed these cells to a variety of anti-HER4 antibodies. Thereby, the group identified anti-HER4 antibodies, amongst them a particular one named "C6", which

mimicked NRG1 mediated effects and showed pro-apoptotic activity characterized by PARP cleavage of Poly(ADP-ribose)-Polymerases (PARP), production of reactive oxygen species (ROS), and depolarization of the mitochondrial membrane. Lanotte et al. localized 4ICD in "C6" treated cells preferably at mitochondria, which suggests a "C6" triggered signaling that prevents translocation of 4ICD into the nucleus and facilitates its interaction with pro-apoptotic molecules. In addition, preclinical in-vivo applications performed by Lanotte et al. revealed reduced growth of appropriate HER4-positive tumor cell xenografts in nude mice. Nevertheless, additional investigations are required to demonstrate the usability of "C6" (or related IgGs) under human like conditions. At this point it is important to note that the use of the native HER4-specific NRG1 by Lanotte et al. showed anti-tumorigenic rather than pro-proliferative effects as observed by Okazaki et al. This apparent contradiction suggests that NRG/HRG triggered effects can differ depending on the cell-specific molecular context, which requires further elucidation.

Worth mentioning is also that an antibody-based HER4 targeting is also subject of investigation within other fields of cancer research such as lung [105] or prostate cancer [108,109].

A recent study impressively demonstrated the clinical implication of the molecular context of HER4 [106]. The researchers recognized the intracellular location and the phosphorylation of YAP, which is critically involved in brain metastasis of BC. They described in detail that pYAP has basically a favorable impact on ER-positive disease, whereas unphosphorylated and cell nucleus-located YAP seems to be very present in primary BCs with brain metastasis. In contrast, nuclear-localized pYAP in ER-negative BC was associated with a rather aggressive clinical behavior, disease relapse in the brain, and shortened patient's survival. (Thus, the formation of brain metastasis as a function of HER4 and HER4-interacting molecules is also relevant insofar, as a number of NRG ligands have been found to be present both in primary BCs [107] and in particular in brain tissues [110]). Even though, the observations made by Kalita-de Croft et al. require further exploration in a preclinical setting the study strongly emphasizes that a single-parametric anti-HER4 targeting is quite likely not sufficiently selective but requires extended companion diagnostics on the molecular level and finally concomitant treatments.

Another (at least theoretical) strategy of HER4 targeting is the use of HER4-specific kinase inhibitors, however, RTK inhibitors most often show cross-specificity or cross-activity (e.g., against RTKs amongst, but

also beyond the EGFR family) [89]. Potentially, a recently synthesized kinase inhibitor with enhanced selectivity for HER4 is more suitable for specific receptor targeting [111], presumed that HER4 takes tumor-promoting effect. However, a kinase inhibitor would most probably not inhibit HER4 receptor cleavage and the phosphorylation-independent signaling of 4ICD.

An approach based on splice-switching oligonucleotides (SSOs), introduced by Nielsen et al. is rather sophisticated and quite HER4-specific [112]. The group used the standard models of HER4/ER double-positive BC cell lines MCF-7 and T-47D and demonstrated a shift of the HER4 receptor expression with CYT1 or CYT2 intracellular domains towards the CYT1 isotype by exposing the cells to SSOs. Due to the CYT1- and CYT2-specific recruitment of downstream signaling molecules this expression-shift caused attenuated PI3K/Akt pathway signaling, whereas the MAPK pathway was not affected. Essentially, the usage of CYT1-promoting SSOs caused a reduced tumor cell growth in-vitro and in a preclinical in-vivo model. Nevertheless, this approach requires also further and deeper exploration in order to evaluate its potential clinical use.

Intriguing is the unorthodox suggestion to apply a native ligand, i.e., NRG4, which exclusively binds to HER4, together with the therapeutic antibodies trastuzumab or pertuzumab for a simultaneous and reinforced anti-HER2 targeting of HER2-enriched BC [70]. In Mianos' pre-clinical study the treatment efficiency was higher when cells were exposed to trastuzumab plus NRG4 or pertuzumab plus NRG4 compared to the administration of anti-HER2 antibodies alone. The authors speculated that a NRG4-induced homodimerization of HER4 receptors might contribute to a growth-inhibitory effect and rather promotes cellular differentiation. This recently conceived idea is basically compatible with previous studies by which an HRG/NGR ligand-caused delay of breast cancer proliferation and enhanced cell differentiation has been observed [113,114].

Finally, molecular biomarkers, which have been found to be essential for the initial HER4 receptor processing and those involved in oncogenic activity of 4ICD represent potential therapeutic targets. TACE, γ -secretase [40,52,53,60,115,116], but also further downstream the mdm2 represent those molecules [117–119]. This is worth to be considered because it has been suggested that 4ICD induces a CDK4/6-dependent phosphorylation of mdmx at Ser314, which facilitates the stabilization of an mdmx/mdm2 molecule complex [119]. This complex might on the one hand suppress the transcriptional activity of p53 but on the other hand also result in an enhanced ubiquitination of p53 and subsequently in proteasomal p53 degradation. This mechanism additionally involves a reduced p21 expression and finally results in a loss of cell cycle control (and together with a decreased presence of p53 in a reduced capacity for apoptotic cell death). Thus, this mechanism presents another way how HER4/4ICD potentially drives cell proliferation (see also Fig. 2). Nevertheless, the usability of the aforementioned signaling axis as a potential target in the context of HER4 expression in (luminal) BC remains to be thoroughly explored yet.

Overall, a variety of approaches have been designed to target HER4 and are being investigated in different pre-clinical, and to some extent in clinical settings. However, individual strategies need to explicitly account for different molecular conditions that affect the mode of action of HER4/4ICD. Those conditions are supposed to be BC subtype-specific. It is essential to specifically address different mechanisms of receptor activation and subsequent signaling, amongst them ligand-dependent vs. independent activation, receptor cleavage, and finally signaling triggered by an activated receptor kinase and/or by the interaction of 4ICD with tumor-promoting or -suppressing signal transducers.

3. Summary and perspectives

Amongst the EGFR-related receptor-tyrosine-kinases the HER4 isotype and its related isoforms play an exceptional role in BC. While a proliferating activity is characteristic for the EGFR, an oncogenic

activity of HER3 and in particular of HER2 is unequivocal. In comparison, both an oncogenic and a tumor-suppressing function have repeatedly been attributed to the HER4 receptor. However, the real impact of HER4 expression and activation on the tumor cells behavior obviously differs in individual BC subtypes and depends on a number of cell extrinsic and intrinsic factors. A tumor suppressing impact has been attributed to HER4 in HER2-positive and triple-negative BC. In estrogen receptor-positive (i.e., luminal) BC, however, the impact of HER4 seems to be quite complex and depends on a number of molecules that affect and determine the HER4 function. Different HER4-specific ligands generate diverse phosphorylation patterns at the receptor level and thus ligand-specific downstream signaling pathways. In addition, a ligand-enhanced two-step HER4 cleavage caused by TACE and γ -secretase entails the release of 4ICD with inherent and unique signaling activity. The intracellular routing of 4ICD towards either cytoplasmic or nuclear cell compartments enables the interaction with molecules, which drive apoptotic cell death, cell differentiation, or cell proliferation. Thus, the prognostic value of HER4 in luminal BC is not only determined by the presence or absence of the receptor itself or the predominance of specific HER4 isoforms but also by molecules that control the intracellular occurrence of 4ICD upon HER4 cleavage and its compartment-specific activity.

Moreover, HER4 seems to have the capacity to affect target-specific tumor cell treatments, amongst them antibody-based anti-HER2 treatments but particularly the endocrine treatment with TAM. Evidence suggests an enhanced efficiency of trastuzumab therapy in HER2/HER4 double-positive tumors compared to HER2-positive/HER4-negative cancers. In contrast, the treatment efficiency with TAM is apparently lower for ER/HER4 double-positive tumors compared to ER-positive/HER4-negative BCs. These observations imply a predictive value of HER4 - in particular in luminal BC - and suggest to evaluate a (concomitant) HER4 receptor targeting for this specific BC subtype in appropriate preclinical in-vitro and in-vivo models. In this context, the relevance of HER4 expression, and in particular of the presence and function of 4ICD-regulating molecules need to be more extensively explored. Finally, the integration of HER4 (and HER4-affecting molecules) into clinico-pathological diagnostics might enhance the stratification of existing BC subtypes and might extend the spectrum of individualized therapy of (luminal) BC patients.

Credit author statement

GB conceptualization and writing of the manuscript.

Declaration of Competing Interest

The author does not declare any potential conflicts of interest.

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