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HER4 Intracellular Domain (4ICD) Activity in the Developing Mammary Gland and Breast Cancer

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Abstract

The HER4 receptor tyrosine kinase was the final member of the EGFR-family to be discovered. In contrast to the other three members of this receptor family which function primarily as mitogenic effectors in the breast, HER4 appears to have multiple divergent functions in the normal and malignant breast. Interestingly, the majority of HER4 activities in the breast including pregnancy induced differentiation and lactation initiation, transcriptional activation, tumor cell proliferation, growth suppression, and induction of apoptosis appear to be mediated by an independently signaling soluble HER4 intracellular domain (4ICD). The 4ICD can accumulate within the nucleus or mitochondria and subcellular localization of 4ICD in part determines the physiological response of breast cells to 4ICD action. Here I will discuss the evidence supporting the role of 4ICD as the critical effector of HER4 signaling in the breast. In addition a developmental and temporal model of 4ICD action in the normal breast and during the progression of breast cancer will be presented to explain the paradox of divergent HER4 and 4ICD activities.

Keywords

EGFR-family; Estrogen receptor; BCL-2 family; Apoptosis; Transcription

Proteolytic Processing of HER4 to Generate a Soluble HER4 Intracellular Domain (4ICD)

It is well accepted that the HER4 transmembrane receptor undergoes regulated intramembrane proteolysis (RIP) at the cell surface to release a soluble intracellular domain (ICD); a property that remains unique among EGFR-family members. An excellent review of the molecular details underlying HER4 RIP was recently published elsewhere [1]. In summary, ectodomain cleavage of the 180 kDa HER4 cell surface receptor is mediated by tumor necrosis factor α converting enzyme (TACE), a member of the ADAM metalloproteinase family referred to as ADAM17 [2]. Following TACE cleavage the ca. 120 kDa HER4 ectodomain, which includes the ligand binding region, is shed into the extracellular milieu while the remaining 80 kDa cleavage product (m80) is retained as a transmembrane peptide. The m80 harbors an active tyrosine kinase [3] and a carboxyl terminus with several potential tyrosine phosphorylation sites. Although the m80 is phosphorylated it remains unclear if this membrane tethered peptide transmits cellular signals. The HER4 m80 however is a substrate for presenilin-dependent - secretase cleavage. This RIP event results in membrane release of a soluble 4ICD. Depending upon specific cellular properties, that we are beginning to understand, membrane released 4ICD may translocate to the nucleus or remain in the cytosol where mitochondrial accumulation has

been observed (Fig. 1). The 4ICD is emerging as a unique independently signaling molecule and the impact of 4ICD signaling in the breast will be discussed in detail here.

Mechanism of HER4 RIP

HER4 RIP is promoted by HER4 binding of a cognate ligand including the HER4 and HER3 ligand heregulin (HRG) or the HER4 and EGFR ligands Hb-EGF and betacellulin [4]. In multiple cell systems transient or stable HER4 overexpression results in ligand independent HER4 RIP [5–9]. Both ligand stimulation and HER4 overexpression results in HER4 phosphorylated activation and in general the levels of HER4 RIP correlate with the levels of HER4 tyrosine phosphorylation. In fact both ligand-dependent and ligand-independent mechanisms of HER4 RIP require a functional HER4 kinase domain [10–12]. The exact contribution of HER4 kinase activity to RIP remains to be determined, but an active HER4 kinase may facilitate recruitment and/or regulate activity of proteolytic complexes. For example the γ -secretase catalytic complex can be regulated by PI3K and MAPK activity [13], two common effectors of an activated HER4 pathway. In addition, and as been shown for the γ -secretase substrate amyloid precursor protein [14], phosphorylation of HER4 may serve to recruit the γ -secretase proteolytic complex. In HEK 293 and T47D cells, HRG drives HER4 accumulation within lipid rafts [15,16] which are also enriched for both TACE and γ -secretase [16–18]. Although there is increasing evidence to suggest that phosphorylation of γ -secretase and its substrate can modulate γ -secretase mediated proteolytic processing, at this time the molecular details remain poorly defined [19]. Nevertheless, a unique function for membrane associated HER4 remains to be confirmed raising the possibility that a major function of ligand activated HER4 is to drive RIP of HER4 to release the potent 4ICD signaling molecule. Therefore deciphering the molecular mechanisms regulating HER4 RIP will be essential for understanding the regulation of 4ICD signal transduction as well as the contribution of cell surface associated HER4.

Once released from cellular membranes 4ICD migrates within the cytosol, localizes at perinuclear regions and through what appears to be a regulated process 4ICD accumulates within nuclear or mitochondrial subcellular compartments. The cellular responses to 4ICD are diverse including proliferation [20–22], mammary epithelial differentiation and lactation [10, 23], activation of gene expression [8,10,21,23], apoptosis [7,11], and cell cycle arrest [12]. Importantly, these cellular responses are in many cases directly associated with a specific 4ICD subcellular context and shuttling of 4ICD between different subcellular compartments may regulate the life or death decisions in a cell. Subcellular localization of 4ICD and subsequent cellular responses are regulated by several 4ICD interacting proteins (Table 1) and 4ICD functional domains (Fig. 2). As discussed herein the contribution of interacting proteins and functional domains to the biological activity of 4ICD has been explored with unexpected results.

Nuclear Translocation of 4ICD

The first description of HER4 nuclear accumulation within human tissues was reported in 1998 by William Gullick and colleagues [24]. These initial immunohistochemical observations were extended to include IHC of primary breast tumors where the same group observed nuclear HER4 immunoreactivity within breast tumors and a small percentage of adjacent normal epithelium [25]. Several lines of genetic and biochemical evidence strongly implicate 4ICD and not full-length HER4 as the predominant and functional form of nuclear HER4. (1) The HER4 isoform, HER4 JM-b, lacking a 12 residue juxtamembrane stretch harboring the TACE cleavage site [26], fails to undergo ligand mediated RIP and nuclear localization [6]. (2) Similarly pharmacological inhibitors of γ -secretase or base substitutions within the HER4 γ -secretase cleavage site abolish HER4 RIP and nuclear localization [7–10,21]. (3) Nuclear

extracts are enriched with 4ICD following ligand stimulation of ectopic expressed or endogenous HER4 [9,21]. (4) Transcriptional coactivation, a functional read-out of nuclear HER4/4ICD activity, is abolished when RIP is prevented [8–10,21]. In addition, binding of HER4 to target promoters by chromatin immunoprecipitation (ChIP) analysis is only detected when using antibodies directed against the carboxyl-terminus which precipitate both full-length HER4 and 4ICD but not an amino-terminal antibody that precipitates HER4 and not 4ICD [9,21]. (5) Finally, a mechanism that describes nuclear translocation of a 185 kDa transmembrane protein remains to be described whereas the 80 kDa 4ICD is within the size range that can be accommodated by the nuclear import pore complex. It should be noted however that the molecular mechanism of 4ICD nuclear import is a critical underexplored area of investigation.

Interestingly a 4ICD isoform referred to as CYT2, which lacks 16 residues including a PI3K binding site (Fig. 2), exhibits enhanced nuclear localization and transcriptional coactivator activity when compared to the full-length CYT1 isoform [27]. The mechanisms that accounts for these differences remains to be determined but may provide clues to the mechanism of 4ICD nuclear import. Also of interest, however, is a recent IHC study using an antibody directed against the HER4 ectodomain which suggests that low levels of intact HER4 were present in a small percentage of human breast tumors [28]. Although IHC experiments are riddled with issues of specificity and sensitivity especially in archived tissue these results underscore the need for a comprehensive examination of HER4 localization including analysis of nuclear extracts from tissues with active HER4 signaling. Because of the difficulty in obtaining sufficient fresh human tumor samples necessary for subcellular fractionation the mouse mammary gland may provide an excellent experimental system to confirm 4ICD nuclear activity in response to developmentally regulated activation of HER4 RIP.

HER4/4ICD is Essential for Mammary Gland Development

Our current view of HER4 activity in the developing mammary gland has been provided through the analysis of several independent genetic and biochemical systems. A seminal study of EGFR-family expression and activation during mammary gland development by Schroder and Lee in 1998 provided the first indication that HER4 may contribute to mammary gland function. Despite low levels of HER4 protein in extracts from virgin, pregnant, and lactating mammary glands a dramatic increase in HER4 phosphorylation, an indication of active receptor signaling, was observed at late pregnancy and during lactation [29].

In direct concordance with HER4 activation during pregnancy and lactation, expression of a dominant negative HER4 mutant driven by the mammary gland specific MMTV promoter resulted in defective lobuloalveolar development observed at 12 days of lactation and impaired expression of the milk genes β -casein and whey acidic protein (WAP) [30]. Consistent with this initial observation, tissue-specific deletion of HER4 within mammary epithelium results in impaired pregnancy and parturition induced epithelial proliferation, disengaged lobuloalveolar distention at late pregnancy and parturition, and ultimately lactational failure (Fig. 3) [23]. Similar results were obtained when HER4 null mice are rescued from embryonic lethality through transgenic expression of HER4 in the developing myocardium [31]. In both genetic models it is clear that loss of HER4 function during pregnancy and parturition is catastrophic as pups born to HER4 deficient dams die from malnutrition [23,31]. The ligand that regulates HER4 activity in the breast remains to be identified. A significant concordance between phenotypes observed in HER4 deficient and HRG α -null mammary glands, however, strongly implicates HRG α as an important regulator of HER4 signaling during pregnancy and at parturition [32].

Interestingly, the phenotype observed in HER4 deficient mammary glands directly overlapped with mammary gland phenotypes reported with STAT5A null mice [33–35]. The concordance between the HER4 and STAT5A null phenotypes was striking and direct coupling of these signaling pathways at late pregnancy and parturition is further supported by the lack of STAT5A phosphorylation at the regulatory Y694 in HER4 deficient mammary glands [23, 31]. Although contrary to dogma, retention of STAT5A activation in prolactin receptor (PrIR) null mammary glands at late pregnancy suggests that HER4 and not PrIR is the obligate regulator of STAT5A mediated lactational initiation at parturition [23]. An intriguing and controversial idea worthy of further investigation.

Nuclear 4ICD Regulates STAT5A Mediated Milk-Gene Expression During Lactation

Detection of HER4 by IHC in the mammary gland during late pregnancy and lactation revealed dramatic nuclear localization within secretory epithelium suggesting that functional activation of HER4 during lactation results in RIP and nuclear accumulation of 4ICD (Fig. 4a) [23]. In fact, during lactation and in breast tumor cells following HRG stimulation we have observed significant nuclear colocalization of 4ICD and STAT5A within secretory epithelium (Fig. 4c,d) [9,23] (unpublished observations) raising the possibility that nuclear 4ICD influences STAT5A activity during lactation. To this end, we have shown that 4ICD liberated from ectopically expressed full-length HER4 directly interacts with STAT5A [9,30] and several laboratories including our own have demonstrated that HER4 dramatically potentiates STAT5A mediated transcriptional activation of the β -casein promoter [8–10,27,36,37]. Significantly, HER4 with transmembrane mutations that abolish γ -secretase mediated RIP failed to coactivate STAT5A in the β -casein promoter assay indicating that 4ICD is required for this activity [8,10]. A role for 4ICD in β -casein regulation is further supported in experiments where an independently expressed 4ICD is sufficient to potentiate β -casein promoter activity [10,27] and stimulate endogenous β -casein expression in the HC11 mouse mammary epithelial cell line [10]. Furthermore, HER4 null mammary glands fail to express the STAT5A regulated milk-genes β -casein and WAP during lactation [23].

Although the exact mechanisms underlying 4ICD and STAT5A coupled transcriptional activities regulating lactation requires further investigation some molecular details have emerged which suggest that 4ICD employs a strategy unique to receptor tyrosine kinases to regulate STAT5A. STAT5A directly interacts with HER4 and 4ICD and this interaction requires both an intact HER4/4ICD kinase domain and a functional STAT5A Src homology 2 (SH2) domain [9,10,30]. SH2 domains mediate specific interactions between signaling molecules and phosphotyrosine residues and the STAT5A SH2 domain mediates interaction between STAT5A and HER4 Y964 [38]. Presumably the HER4 kinase domain has a dual role in this process to: (1) promote 4ICD phosphorylation thereby providing a STAT5A docking site [9,10,30] and (2) facilitate HER4 RIP [10,11]. Once liberated from the cell membrane the 4ICD/STAT5A complex cotranslocates to the cell nucleus where we have shown by ChIP in the T47D breast cancer cell line that both endogenous proteins bind to STAT5A target sites within the β -casein promoter [9] and 4ICD promotes β -casein expression in mouse mammary epithelial HC11 cells [10]. Most interesting however is that the 4ICD/STAT5A transcriptional complex is excluded from the nucleus and STAT5A mediated gene expression is abolished when the 4ICD intrinsic NLS is inactivated [9]. Similarly, STAT5A accumulates in the cytosol and expression of the STAT5A target genes β -casein and WAP are suppressed in HER4 null lactating mammary glands [23]. Taken together, these novel observations suggest that 4ICD regulates STAT5A transactivation of milk-gene expression in part by functioning as a nuclear chaperone for STAT5A. Despite compelling data to support this model the contribution of 4ICD to STAT5A mediated gene expression and lactation initiation requires in vivo confirmation using genetic models that for example abolish HER4 RIP and/or 4ICD nuclear

translocation. Equally important are future experiments designed to decipher the influence of nuclear 4ICD on the molecular regulation of transcription including promoter association, coregulator recruitment, and chromatin dynamics. For example, functional confirmation of reports describing 4ICD intrinsic transactivation activity [7,9,27,39] would be a strong foundation to build this exciting and important area of research.

HER4/4ICD and ER Crosstalk in Breast Cancer

Given the important contribution of EGFR-family members to breast cancer and the essential role for HER4 during breast development there has been considerable interest in determining the contribution of HER4 expression to breast tumorigenesis. The results and conclusions from multiple HER4 clinical studies are discussed elsewhere in this issue. Here I will focus my discussion on the contribution of 4ICD signaling to breast tumorigenesis.

In multiple clinical studies of HER4 expression in primary breast tumors a significant association between HER4 expression and positive ER status is consistently reported [20,40–42]. Indeed, in one study over 90% of ER (+) breast tumors were found to also express HER4 [43]. Significantly, HER4 has emerged as a potential estrogen regulated gene in both breast tumors [44] and breast cancer cell lines [21]. In fact the HER4 promoter harbors three putative estrogen response element (ERE) half-sites and ChIP analysis indicates that estrogen recruits ER α to a chromatin region containing one of these sites [21]. Thus in human breast tumor cells there appears to be a strong selection for ER α and HER4 coexpression that is maintained by circulating estrogens.

Experiments designed to examine a potential functional relationship between HER4 and ER α have generated surprising results. The laboratory of Marc Lippman used a ribozyme strategy to suppress HER4 expression in the HER4/ER(+) T47D and MCF-7 breast cancer cell lines. Although HER4 expression in breast tumors is associated with a less aggressive non-proliferative and differentiating tumor phenotype [69], ribozyme suppression of HER4 expression significantly curtailed T47D and MCF-7 colony formation in soft-agar and xenograph tumor growth [45,46] implicating a proliferative role for HER4 in breast tumor cells. Similarly, using an RNAi strategy to suppress HER4 expression we observed impaired estrogen stimulated growth of both T47D and MCF-7 cell lines [21] (unpublished observations). Likewise, increased colony formation of MCF-7 cells with ectopic HER4 expression required exogenous estrogen [20]. Clearly in these experimental systems HER4 promotes growth of ER α (+) breast tumor cells and the ability of HER4 to promote breast tumor cell growth is dependent upon exogenous estrogen.

Apparently the growth promoting effects of HER4 in human tumor cell lines requires cross-talk with ER α signaling. This cooperation between HER4 and ER α to promote tumor cell proliferation most likely provides the selective advantage for ER α and HER4 coexpression in human breast tumors. Moreover these results raise the possibility that circulating estrogens promote tumor cell growth by maintaining HER4/ER α crosstalk. Indeed, we and others have shown that HER4 is an estrogen regulated gene [21,44] and we have shown that estrogen establishes a growth-promoting HER4/ER α autocrine loop in breast tumor cells [21]. This is supported in part in the developing mammary gland where HER4 contributes to pregnancy induced mammary epithelial proliferation, a phenotype that overlaps with the lobuloalveolar proliferation and expansion defects observed in ER α null epithelium [47]. One strong prediction based upon this hypothesis is that strategies to uncouple HER4 and ER α signaling may have therapeutic benefits. To this end we have begun to decipher the molecular basis of HER4 and ER α crosstalk in breast cancer.

Nuclear 4ICD Functions as a Growth Promoting Estrogen Receptor Coactivator

Because of the obvious clinical implications, crosstalk between members of the EGFR-family and ER α in breast cancer has been an area of intense investigation and many important findings have been highlighted elsewhere [48]. In summary, EGFR-family members may indirectly impact ER α signaling through mechanisms that involve phosphorylation of ER α or ER α coactivators. Likewise, ER α may establish an autocrine loop in EGFR expressing tumors cells through the upregulation of EGF-family ligands. Although similar mechanisms may regulate HER4 and ER α crosstalk, our findings suggest a completely novel mechanism of crosstalk between a cell surface and nuclear receptor involving direct regulation of ER α genomic activity by 4ICD.

The ability of 4ICD to coactivate STAT5A and the correlation between HER4 and ER α expression in breast cancer led us to investigate 4ICD as a potential ER α coactivator. Indeed, when cotransfected with a minimal estrogen response element (ERE) fused to a luciferase reporter, HER4 significantly potentiated estrogen regulated expression from the reporter construct [21]. In a similar experiment, stable overexpression of HER4 in the MCF-7 cell line also increased estrogen stimulation of ERE reporter activity [20]. Treatment of transfected cells with the pure anti-estrogen ICI which not only occludes estrogen binding but results in degradation of ER α , abolished HER4 stimulation of estrogen activity indicating that HER4 cooperates with ER α to stimulate gene expression [21]. These results were substantiated when suppression of HER4 expression in both MCF-7 and T47D cells demonstrated that HER4 was an obligate endogenous coactivator for the estrogen regulated genes progesterone receptor (PgR) and stromal cell-derived factor 1 (SDF-1), whereas expression of another estrogen regulated gene, trefoil factor 1 precursor (pS2) was unaffected by the loss of HER4. These results represent the first description of a cell surface receptor directly regulating the genomic activity of a nuclear receptor and the following additional experimental evidence further implicate 4ICD as the potent ER α coactivator in this system: (1) the HER4 processing mutant V673I, which retains normal signaling activities associated with cell surface HER4, fails to undergo RIP to release 4ICD [8] and lacks the ability to coactivate ER α gene expression and promote estrogen stimulated proliferation; (2) similar to several other ER α coactivators described to date estrogen stimulates a physical interaction between 4ICD, but not the HER4 ectodomain, and the ER α domain E harboring the ER α ligand-dependent activation function 2 (AF2) [21]; (3) estrogen stimulates nuclear localization of endogenous 4ICD and subsequent binding of 4ICD with ER α at PgR and SDF-1 target promoters [21]; and (4) independently expressed 4ICD but not the HER4 ectodomain potentiates estrogen stimulated ERE activity at levels equivalent to full length HER4 (unpublished observations).

Although these preliminary results are intriguing the molecular basis of 4ICD coactivator and tumor cell proliferation functions remain to be deciphered. For example, nuclear 4ICD promotes estrogen regulated proliferation in the absence of exogenous HER4 ligand. Although basal levels of 4ICD are observed in breast tumor cell lines we have observed 4ICD accumulation in response to estrogen (unpublished observations) suggesting that estrogen itself promotes HER4 RIP. Another possible mechanism of 4ICD generation in the absence of exogenous HER4 ligand was provided by a recent elegant study demonstrating that HER4 RIP to generate a nuclear 4ICD requires matrix metalloproteinase 7 (MMP7) cleavage of the HER4 ligand Hb-EGF [22]. MMP7 generates a soluble Hb-EGF harboring the HER4 binding motif which in turn stimulates HER4 RIP and promotes release of 4ICD. Importantly, both MMP7 and an independently expressed 4ICD (CYT2 isoform) promote tumor formation of untransformed mouse epithelial cells. The growth promoting activity of 4ICD in this system appeared to be mediated by nuclear signaling activity [22]. The molecular basis of nuclear

4ICD proliferative activity remains unclear nevertheless results from our laboratory and others suggest that 4ICD coactivation of ER α plays an important role.

Interestingly, 4ICD functions as a selective ER α co-activator binding to the promoters and regulating expression of PgR, SDF-1, and HER4 itself but not pS2 [21]. Furthermore, pS2 expression is regulated through a canonical ERE whereas 4ICD is recruited with ER α to an alternate ERE composed of an ERE half site and associated SP-1 or AP-1 sequence element. The laboratory of Powell Brown has made significant progress towards clarifying the molecular basis of estrogen regulation at AP-1 sites and his group has examined the consequences of suppressed estrogen stimulation of genes regulated by these promoters. To summarize the findings of his laboratory and others, estrogen stimulates ER α recruitment to AP-1 sites within target promoters, even in the absence of an obvious ERE [49–52], and surprisingly these AP-1 target genes harboring non-canonical ER α recruitment sequences, including cyclin D1 and c-myc, mediate estrogen induced proliferation of breast tumor cells [52,53]. Interestingly, ER α does not directly bind DNA at these sites but rather is thought to be part of a coactivator complex that is anchored by the c-Jun transcription factor [53]. Both suppression of HER4 expression and expression of a dominant negative c-Jun (Tam67) results in a significant but not complete loss of estrogen proliferative response in breast cancer cells [21,45,52]. It is therefore tempting to speculate that 4ICD is an important component of the growth promoting ER α coactivator complex at AP-1 sites in estrogen regulated genes. With AP-1 sites emerging as one of the predominant sequence elements involved in estrogen stimulated ER α recruitment [51] and estrogen stimulated proliferation of breast tumor cells [52,53] the exact composition of coactivation complexes at these promoters will be an area of important future investigation.

Molecular Basis of 4ICD Coactivator Function

Despite compelling evidence that 4ICD functions as a transcriptional coactivator regulating the growth and differentiation of breast epithelium the exact contribution of 4ICD to gene expression at target promoters remains to be identified. When fused to the GAL4 DNA binding domain 4ICD promotes transcription in a reporter gene assay [7,9,27,39] raising the possibility that 4ICD harbors independent transactivation activity. This putative intrinsic transactivation activity, however, still requires confirmation. Alternatively 4ICD may function as an adaptor protein to facilitate recruitment of transcriptional coactivators to target promoters. For example when ectopically co-overexpressed, 4ICD has been shown to interact with the Yes-associated protein (YAP) transcriptional coactivator and enhance expression from a reporter gene [39, 54]. It will be interesting to see if this potent transcriptional complex contributes to gene expression at physiologically relevant 4ICD target promoters. Likewise there is a formal possibility that the transactivation activity of STAT5A, by virtue of its interaction with 4ICD, is corecruited to estrogen response genes.

Alternatively recent evidence indicates that 4ICD may regulate gene expression by interacting with and modulating the activity of transcriptional corepressors. For example, the carboxyl-terminal region of 4ICD interacts with the ETO2 transcriptional repressor and derepresses transcription from a GAL4 reporter [55]. Although confirmation in a physiological setting is necessary, these results raise the possibility that nuclear 4ICD regulates transcription by reversing the activity of transcriptional repressors. In contrast, in the developing brain HER4/4ICD interacts with the TAB2/N-CoR transcriptional repressor complex and suppresses astrocytic gene expression in a HER4 RIP dependent manner [56]. The molecular basis of this transcriptional repressor function for 4ICD remains unclear but may be specific for neurogenesis. Finally, 4ICD has been shown to interact with MDM2 and partially reverse MDM2 repression of p53 transcriptional activity [57].

Although substantial evidence supports 4ICD function as a transcriptional coactivator it is clear that additional experiments are required to decipher the exact mechanism of 4ICD action. With several estrogen regulated 4ICD target promoters identified to date and the impact of 4ICD on estrogen stimulated proliferation of breast cancer cell lines already established experimentally, the impact of 4ICD on global estrogen stimulated gene expression would be an important and logical starting point. Numerous ER α coactivators have already been identified which contribute divergent activities at target promoters through complex interactions with ER α . Interestingly, 4ICD harbors two potential nuclear receptor (NR)-boxes (aka LXXLL domains), a motif shared by nuclear receptor coactivators that mediate interactions between coactivators and their partner receptor. We are currently exploring the contribution of the 4ICD NR-boxes to ER α coactivation but one possibility is that through its interaction with ER α , 4ICD may function as an adaptor protein facilitating recruitment and/or stabilizing coactivator complexes at estrogen response promoters. In addition, ectopically expressed 4ICD harbors a constitutively activate kinase domain [3] and nuclear 4ICD may therefore influence transcription through phosphorylated activation of additional coactivators or ER α itself. An analysis of 4ICD influence on the assembly of estrogen regulated coactivator complexes and chromatin dynamics should provide important insights into both 4ICD nuclear functions and estrogen regulation of breast tumor proliferation.

Cytosolic 4ICD Promotes Apoptosis of Breast Tumor Cells

In addition to membrane and nuclear localization of HER4/4ICD in human breast tumor cells some reports indicate that 4ICD also localizes within the cytosol of tumor cells [11,28,58]. Although cytosolic 4ICD observed in tumor sections may represent a transient population fixed and “captured” during translocation to the nucleus, the majority of tumors staining for cytosolic HER4/4ICD lack detectable nuclear 4ICD [11] raising the possibility that cytosolic 4ICD is a functionally distinct population.

Our laboratory explored the possible function of a cytosolic 4ICD and obtained exciting and unexpected results. As discussed, evidence from several laboratories indicates that estrogen stimulation of HER4/ER α (+) breast tumor cells results in nuclear translocation of the 4ICD coactivator, 4ICD dependent activation of estrogen response genes, and breast tumor cell proliferation. Stimulation of the same cells however with the HER4 ligand HRG results in pronounced tumor cell killing [7,11]. Paradoxically both the proliferative effects of estrogen and HRG induced cell-killing are mediated by 4ICD and these divergent activities require HER4 RIP to release 4ICD [7,8,11,21]. However 4ICD cell-killing occurs independent of 4ICD nuclear activity [8] suggesting that a cytosolic 4ICD population directly influences a cell suicide program.

Surprisingly, we found that in response to HRG both MCF-7 and T47D cells accumulate 4ICD within the mitochondria and endoplasmic reticulum [8,11]. Both organelles represent important sites of cellular apoptosis initiation and regulation [59]. Furthermore, 4ICD harbors a cell-killing or BH3 domain that is characteristic of a family of apoptosis initiators of the BCL-2 family referred to as BH3-only proteins. We have shown that 4ICD possesses many of the characteristics of BH3-only protein including (1) BH3-domain dependent cell-killing activity, (2) the 4ICD BH3-domain models an amphipathic helix, a conformation essential for BH3-domain activity, (3) 4ICD accumulates with mitochondria and endoplasmic reticulum following apoptotic stimulus, (4) 4ICD apoptotic activity is inhibited by the broad spectrum caspase inhibitor zVAD, (5) 4ICD interacts with and its apoptotic activity is inactivated by anti-apoptotic BCL-2 which is known to sequester and inactivate BH3-only proteins, (6) and 4ICD initiates apoptosis through activation of the mitochondrial dysfunction gatekeeper protein BAK [8,11]. Regulation of cell death by the BCL-2 family of pro- and anti-apoptotic proteins is a complex process and additional experiments are required to confirm the exact role

of 4ICD in this process. Nevertheless, clinical analysis of primary breast tumors implies an important physiological contribution of cytosolic 4ICD to breast tumor apoptosis. For example, we have shown that accumulation of cytosolic 4ICD, but not membrane associated HER4, is significantly associated with apoptotic cells in primary breast tumors [11]. Although this study has limited power due to a small sample size we have recently extended this study to include over 900 primary breast tumors and observed a similar significant association between cytosolic 4ICD and tumor apoptosis (unpublished observations).

Hypothesis: Nuclear and Cytosolic Shuttling of 4ICD Influences Breast Development and Tumorigenesis

The multiple divergent activities of HER4 and 4ICD poses a biological conundrum that has created considerable controversy in the field and confusion for the uninitiated. In an attempt to clarify the contribution of HER4 to breast development and tumorigenesis, I have developed a hypothesis driven model suggesting that the multiple functions of HER4 can in part be explained through the temporal and spatial regulation of 4ICD activity in the developing breast and during breast tumor progression (Fig. 5).

In the developing breast nuclear 4ICD activity regulates epithelial differentiation and lactation at parturition through coactivation of STAT5A. Nuclear 4ICD is most likely generated through HRG α stimulation of HER4 RIP, although evidence suggests that Hb-EGF may also supply this activity [22,60]. During the initiation of breast cancer epithelial cells adapt an ER α (+) phenotype thereby providing a distinct growth advantage. In this context, ER α commandeers the estrogen regulated 4ICD coactivator and establishes a 4ICD dependent growth promoting autocrine loop in the developing tumor driven by circulating estrogens. Thus the role of nuclear 4ICD shifts from a benign STAT5A coactivator driving differentiation in the normal breast to an ER α coactivator promoting breast tumor proliferation. There is a strong selection for the ER α /4ICD signaling axis in developing tumors with several studies reporting between 74–91% of low grade ER α (+) tumors coexpressing HER4 [43,45,61]. Clinically this combination of ER α expression and nuclear accumulation of the 4ICD/ER α transcriptional complex predicts worse patient prognosis than the combination of ER α and membrane associated HER4 [20, 28] providing strong clinical support for a ER α /4ICD tumor cell proliferation signal.

During progression to a more aggressive high grade tumor, however, loss of both ER α and HER4 expression is observed clinically [20,58,62,63]. Presumably, and for reasons that remain poorly understood, ER α proliferative signaling is being supplanted by more potent oncogenic signals. This is also accompanied by a loss of HER4 expression which may be a consequence of disengaged estrogen regulated HER4 expression in the absence of ER α . An alternative and intriguing explanation based upon our work suggests that in the absence of ER α , 4ICD, which is no longer tethered within the nucleus, accumulates within mitochondria where it induces tumor cell killing. In this model 4ICD would function as a biological sensor for ER α activity and apply a brake to tumor progression in the absence of ER α . Thus to overcome this roadblock and progress to a more aggressive ER α (-) phenotype, a successful tumor must actively suppress HER4 expression.

There are three strong predictions based upon this model each with some clinical support. First, disruption of the ER α /4ICD transcriptional complex, for example through endocrine therapy, will result in tumor cell apoptosis and by extension HER4 expression will emerge as an important marker for patient response to endocrine therapy. Indeed, in a survey of patients treated with tamoxifen monotherapy HER4 expression is an independent marker for patient response [64] and the molecular basis of this improved patient response may be related to the cell killing activity of 4ICD. For example, tamoxifen suppresses tumor growth in part by disrupting ER α and coactivator complexes. In submitted work we show that tamoxifen disrupts

the ER α /4ICD transcriptional complex leading to 4ICD BH3-domain dependent tumor cell apoptosis. Second, a successful tumor must evade 4ICD cell-killing activity through a mechanism that suppresses HER4 expression. Another recent clinical study has identified a polymorphism within the HER4 promoter that exhibits suppressed promoter activity and is associated with increased risk for breast cancer [65]. Finally, one would predict that reactivation of HER4 expression in aggressive breast tumors would be a novel therapeutic approach to suppress growth of these tumors. We currently have a clinical trial underway to test this prediction and should have results in the coming year.

Conclusions

Increasing evidence implicates HER4 as an important player during breast development and breast cancer. A plethora of divergent activities have been attributed to HER4 signaling in the breast and many of these are regulated by an independently signaling 4ICD. In fact, 4ICD mediates HER4 activities that are completely novel for a cell surface receptor including regulation of tumor cell proliferation by direct modulation of nuclear receptor genomic activity and induction of apoptosis as a BH3-only protein member of the BCL-2 family. As predicted based upon these unique 4ICD functions, tumor expression of 4ICD in different subcellular compartments has dramatically different effects on breast cancer patient outcome with nuclear 4ICD associated with worse patient outcome. Future studies of 4ICD influence on mammary development and breast cancer will need to focus on both the molecular mechanisms of 4ICD action and specific mechanisms that regulate 4ICD subcellular localization. Manipulation of 4ICD localization and specifically nuclear cytosolic shuttling may represent an effective therapeutic strategy to disengage tumor cell proliferation with fortuitous activation of 4ICD tumor cell killing activity.

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Abbreviations

4ICD	HER4 intracellular domain
TACE	tumor necrosis factor α converting enzyme
RIP	regulated intramembrane proteolysis
ICD	intracellular domain
HRG	heregulin
TF	transcription factor
MMTV	mouse mammary tumor virus
WAP	

	whey acidic protein
SH2	src homology 2
STAT5	signal transducer and transactivator 5
ChIP	chromatin immunoprecipitation
NLS	nuclear localization signal
PgR	progesterone receptor
SDF-1	stromal cell-derived factor 1
ERα	estrogen receptor alpha
ERE	estrogen response element
pS2	trefoil factor 1 precursor
BH3	BCL-2 homology 3

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Figure 1.

Model of HER4 regulated intramembrane proteolysis (RIP). Ligand activated HER4 is proteolytically processed at the cell surface through the sequential activities of TACE and γ -secretase. TACE cleavage is prevented in a natural isoform of HER4 referred to as JM-b which lacks the extracellular TACE cleavage site [26]. Cleavage by γ -secretase is prevented by introducing a transmembrane valine base substitution at position 673 [8] or 675 [10]. The HER4 intracellular domain (4ICD) may remain in the cytosol where it accumulates within the ER and mitochondria and regulates apoptosis [8,11]. Alternatively 4ICD may accumulate within the nucleus, interact with transcription factors (TFs) at target promoters, and coactivate gene expression [9,21].

Figure 2.
4ICD functional domains and interacting proteins. Details are described in text.

Figure 3.

HER4 regulates lobuloalveolar development and epithelial differentiation at parturition. **a, b** Whole-mounts of carmine stained mammary glands from biparous *HER4*^{+/+}WAP-Cre (**a**) and *HER4*^{Flox/Flox}WAP-Cre mice (**b**) at parturition (L1). Alveolar clusters are indicated by arrows. Note condensed lobuloalveoli in *HER4* deficient mammary glands (**b**). **c, d** Histological analysis of hematoxylin/eosin stained paraffin embedded biparous *HER4*^{+/+}WAP-Cre (**c**) and *HER4*^{Flox/Flox}WAP-Cre (**d**) mammary glands at parturition. Abnormal secretory activity is indicated by the accumulation of luminal lipids in *HER4*^{Flox/Flox}WAP-Cre mammary glands at parturition (arrow in **d**). **e, f** Immunohistochemical staining for the mammary differentiation marker Npt2b in paraffin embedded biparous *HER4*^{+/+}WAP-Cre (**e**) and *HER4*^{Flox/Flox}WAP-Cre (**f**) mammary glands at parturition. *HER4* deficient secretory epithelium fail to undergo terminal differentiation and express Npt2b.

Figure 4.

HER4/4ICD and activated STAT5A colocalize within nuclei of breast epithelial cells. **a, b** Immunohistochemical localization of **(a)** HER4/4ICD and **(b)** activated STAT5A within nuclei of lactating breast epithelium. **c, d** HER4/4ICD and STAT5A colocalize within the nuclei of transfected MCF-7 cells following HRG stimulation. **c** Merge of HER4/4ICD (green) and STAT5A (red) expression in mock stimulated MCF-7 cells. Nuclei (blue) are counterstained with Hoechst. **d** HRG stimulation of HER4 results in dramatic nuclear colocalization of 4ICD and STAT5A. A pixel correlation algorithm is used to quantitate the levels of colocalization with red signaling indicating 100% colocalization and blue indicating 0% colocalization.

Figure 5.

Hypothesis driven model illustrating that the multiple functions of HER4 can in part be explained through the temporal and spatial regulation of 4ICD activity in the developing breast and during breast tumor progression.

Table 1

4ICD interacting proteins.

Interacting protein	Function	References
BCL-2	Suppressor of apoptosis	[11]
ER α	Nuclear receptor; transcription factor	[21]
ETO2	Transcriptional repressor	[55]
MDM2	Negative regulator of p53; ubiquitin ligase	[57]
PI3K (p85)	Membrane signaling complex	[66]
PSD95	Synapse associated scaffolding complex	[67]
STAT5A	Transcription factor; mammary differentiation factor	[30]
TAB2	Signaling protein	[56]
WWOX	Tumor suppressor gene; dehydrogenase/reductase	[68]
YAP	Transcription factor	[39]