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The ERBB3 receptor in cancer and cancer gene therapy

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Abstract

ERBB3, a member of the epidermal growth factor receptor (EGFR) family, is unique in that its tyrosine kinase domain is functionally defective. It is activated by neuregulins, by other ERBB and nonERBB receptors as well as by other kinases, and by novel mechanisms. Downstream it interacts prominently with the phosphoinositol 3-kinase/AKT survival/mitogenic pathway, but also with GRB, SHC, SRC, ABL, rasGAP, SYK and the transcription regulator EBP1. There are likely important but poorly understood roles for nuclear localization and for secreted isoforms. Studies of ERBB3 expression in primary cancers and of its mechanistic contributions in cultured cells have implicated it, with varying degrees of certainty, with causation or sustenance of cancers of the breast, ovary, prostate, certain brain cells, retina, melanocytes, colon, pancreas, stomach, oral cavity and lung. Recent results link high ERBB3 activity with escape from therapy targeting other ERBBs in lung and breast cancers. Thus a wide and centrally important role for ERBB3 in cancer is becoming increasingly apparent. Several approaches for targeting ERBB3 in cancers have been tested or proposed. Small inhibitory RNA (siRNA) to ERBB3 or AKT is showing promise as a therapeutic approach to treatment of lung adenocarcinoma.

Keywords

ERBB3; cancer biology; cancer therapy

Introduction

The epidermal growth factor receptor (EGFR) (ERBB1, HER1), a tyrosine kinase, is evolutionarily ancient and is widely expressed.[1] Additional ERBB family members, ERBBs 2–4, have evolved from EGFR in mammals to establish functionality dependent on receptor interactions. Complex multilayered signaling generated by receptor cross talk and lateral signaling is becoming evident within these family members. Further complexity is imposed by a multiplicity of ligands: epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin, epiregulin, betacellulin, heparin-binding EGF and epigen are known ligands for EGFR. Neuregulins (NRG, HRG) are a family of ligands for ERBB3 and ERBB4. Regulated signaling by these multiple ligand and receptor components is implicated for the maintenance of cell division, proliferation, differentiation, migration and other normal cellular processes. However, deregulated, aberrant signaling due to mutation, amplification and

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All genes and proteins are named by the symbols designated by the Human Genome Organization (HUGO) Gene Nomenclature Committee. They are present in all capital letters, unless specified in reference to a rodent gene/protein. The protein sequence numbering for ERBB3 is based on that presented in the National Center for Biotechnology Information (NCBI) website for human ERBB3.

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presence of active autocrine loops may participate in development of cancer and other diseases. Recent reviews are available covering activation, interaction and signaling of ERBB family members.[2–8]

Attempts are already in progress in the clinic to utilize the EGFR and ERBB2 as molecular targets for cancer therapy. EGFR is being targeted with the monoclonal antibody cetuximab and with two low molecular weight tyrosine kinase inhibitors, gefitinib and erlotinib, with success against several types of epithelial cancers, including head and neck, pancreatic, colorectal and a subset of nonsmall cell lung cancers with mutant or highly expressed EGFR.[9] ERBB2 has been successfully targeted by the monoclonal antibody trastuzumab (herceptin) in breast cancers, where it is often overexpressed and this approach is now used clinically.[10] However, trastuzumab had little or no effectiveness against cancers of the prostate,[11] pancreas,[12] colon and rectum[13] or lung epithelia.[14]

High expression of ERBB3 in certain human cancers led early to the suggestion that it could be a therapeutic target.[15] Nevertheless efforts at targeting ERBB3 in cancers have lagged behind, due in part to its impaired kinase activity; a mainly modulatory role is often assumed, secondary to ERBB2 as 'the master positive regulator of the ERBB network'.[6] However, cross talk among the ERBB receptors that amplifies and diversifies signaling is emerging as a central feature of cancer cells, and in this context ERBB3 can be of key importance. Recent evidence that ERBB3 is responsible for tumor resistance to therapeutic agents targeting EGFR or ERBB2 has illuminated its critical role in cancer.[16] Here, we have reviewed the characteristics of ERBB3 and its potential role in several types of cancer, and illustrate that it is a potential target for siRNA-based therapy in lung cancer.

The *ERBB3* gene and gene expression

Salient features of the ERBB3 gene, mRNA and protein are summarized in Table 1. ERBB3 maps to human chromosome 12q13.2, is 23.2 kb in size and consists of 28 exons[17–19] (NCBI Gene ID 2065, Oct 25, 2006). The four *ERBB* receptor genes are thought to have evolved from a single ancestral gene, with an intermediate progenitor for *EGFR* and *ERBB2* and another progenitor for *ERBB*s *3* and *4*.[20] The gene and protein sequences for the extracellular ligand-binding domain of ERBB3 have 43–45% homology with EGFR and ERBB2 and 56–67% with ERBB4; the cytoplasmic tyrosine kinase domain sequences have 60–63% homology with those of each of the other ERBB receptors.[17,21]

The human *ERBB3* gene is transcribed as a 6.2 kb message of 4080 nucleotides and 1342 codons specifying the full-length protein.[17] There are several *ERBB3* truncated transcripts. A 1.4 kb transcript codes for the first 140 amino acids of extracellular domain I followed by 43 unique amino acids.[22] This transcript is widely expressed in normal and neoplastic cells, with its level relative to the main 6.2 kb message being higher in cell lines with relatively low *ERBB3* expression.[22–24] It transcribed a 24 kDa protein[23] which in mammalian cells formed an intracellular 58 kDa glycosylated dimer that did not appear to bind ligand.[24] The potential functions of this intracellular ERBB3 form remain to be determined.

There are four additional alternate transcripts of 1.6, 1.7, 2.1 and 2.3 kb generated by intron read through.[23] At least three of these code for truncated, secreted sERBB3.[23,25] A p45 sERBB3 consists of extracellular domains I and II and part of domain III, plus 2 unique C-terminal amino acids. A p85-sERBB3 is formed by domains I, II and III and part of IV, with addition of 24 unique C-terminal amino acids. Both forms, and especially the p85 sERBB3, bound NRG and reduced NRG activity as a ligand on breast carcinoma cells.[25] Thus these sERBB3 forms may be potential negative regulators of NRG. In contrast, a p45 form, designated MDA-BF-1, is a putative prostate cancer bone metastasis factor.[26]

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ERBB3 mRNA is present from the earliest stages of development, being detected throughout spermatogenesis,[27] in the nucleus of ejaculated human sperm[28] and in bovine oocytes at all stages.[29] Erbb3 was expressed and active in epithelial cells of mouse uterus during implantation[30] and likewise *ERBB3* mRNA was detected in both the cyto- and syncytiotrophoblast at the time of implantation in the rabbit, with a pattern distinct from those of EGFR, ERBB2 and ERBB4.[31] Similarly during organogenesis *ERBB3* mRNA levels and distribution were distinct from those of other ERBB receptors, suggesting unique functions, as for example in the development of murine teeth[32] and of fetal rat brain.[33,34] In human fetuses *ERBB3* transcripts were detected in liver, kidney and brain but not in heart or lung fibroblasts.[17] ERBB3 is widely expressed in human adult tissues, consistently detected in brain, spinal cord, liver, prostate, kidney and lung (www.genecards.org).

Relatively little is known about regulation of *ERBB3* transcription. The *ERBB3* promoter region is GC rich (65%) and, like EGFR, does not contain a TATA box; there are several transcriptional start sites.[35] Five potential nuclear factor-binding sites were identified and AP2-1 (OB2-1) was implicated in human breast carcinoma cells with high expression of ERBB3 protein.[35] These investigators looked for but did not find evidence for Sp1 transcription factor binding or for upstream or intron 1 enhancers. Involvement of AP transcription factors was confirmed by demonstration that overexpressed AP-2α, AP-2β or AP-2γ in AP-2 deficient HepG2 cells transactivated the *ERBB3* promoter.[36] AP-2γ protein correlated positively and strongly with *ERBB3* mRNA level in breast cancer cells and in SV40 transformed lung fibroblasts, whereas there was a very low or undetectable level of AP-2γ in ERBB3 nonexpressing benign breast epithelial cells and in normal lung fibroblasts.[37] In the latter study, cotransfection experiments indicated that AP-2γ transactivated the *ERBB3* promoter in an *AP2* and *ERBB3* nonexpressing breast cancer cell line. Dominant negative AP-2 suppressed *ERBB3* promoter activity and also downregulated endogenous *ERBB3* mRNA level, to result in decreased proliferation and reduced colony formation in SV40 transformed lung fibroblasts. Whether AP-2 is a main regulatory factor for *ERBB3* transcription in other cell types should be studied.

Estrogen negatively regulated *ERBB3* mRNA levels in estrogen receptor-positive ZR75-1[38] and MCF7[39] human mammary carcinoma cell lines.

ERBB3 expression has recently been found to be regulated by $\alpha_6\beta_4$ integrin in breast carcinoma cells,[40] evidently by effects on translation. The presence of the $\alpha_6\beta_4$ integrin markedly enhanced levels of ERBB3 and phosphotidyl inositol 3-kinase (PI3K)/Akt signaling in MCF7 and MDA-MD-435 cells, while having no effect on ERBB2.

The ERBB3 protein

Primary and crystal structure

The ERBB3 receptor consists of an extracellular ligand-binding domain followed by a transmembrane spanning helix and an intracellular cytoplasmic kinase domain that is flanked by juxtamembrane and C-terminal regulatory regions. It is a glycoprotein of 180 kDa with 10 potential N-linked glycosylation sites; up to 30% of the apparent molecular weight of ERBB3 consists of glycosyl groups. Only one glycosylation site is conserved in all ERBB family members, suggesting that glycosylation patterns may contribute to the unique functioning of each receptor.[41] Glycosylation site Asn[414] was in fact found to be critical to regulation of ERBB3 function: mutation of this site to Gln, in constructs expressed in CHO cells, resulted in autodimerization and heterodimerization with ERBB2 in the absence of ligand, and enhancement of the neoplastic properties of the cells.[42]

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The extracellular domain—As in other members of the ERBB family, the extracellular domain of ERBB3 consists of four subdomains, I (L1), II (C1), III (L2) and IV(C2). Domains I and III (ligand binding) have β-helical folding, while domains II and IV (cysteine rich) have an extended structure held together by disulfide bonds. The I/II and the III/IV sequences probably evolved by gene duplication.[41] Considerable information has been gleaned from X-ray crystallography studies of the extracellular domain structures of ERBB family members. In the absence of ligand, a direct intramolecular interaction between domains II and IV, involving a β-hairpin loop of residues 242–259 in domain II, keeps the ERBB3 in a closed (locked or tethered) conformation that prevents interaction between domains I and III.[43,44] This conformation disrupts the ligand-binding pocket and buries the dimerization arm of domain II. Similar locked conformations have been observed for unliganded EGFR and ERBB4.[1,45] In the presence of ligand, the I and III domains of EGFR are held in a rigid conformation and the putative dimerization loop from domain II extends and interacts intramolecularly with another ligand-bound monomer to form dimers. Mathematical and biochemical modeling studies indicated that another binding event, in addition to ligand binding, is required to explain the observed shape of Scatchard binding plots for EGF/EGFR interactions.[46,47] Most recently, small-angle X-ray scattering methodology confirmed extension of the extracellular domains of both EGFR and ERBB3 upon ligand binding, and suggested that multiple weak interactions over several parts of these proteins contribute to the tethered conformation.[48]

Unique features of the extracellular domain of ERBB3 may give clues as to its special functions. (1) Domain I is a major contributor to ligand binding by ERBB3,[49,50] in contrast to EGFR, where domain III ligand binding is dominant. (2) ERBB3 has higher affinity for NRG, compared with affinity of EGFR for EGF and this is greatly increased by dimerization with ERBB2.[50] (3) In EGFR, ligand-binding sites in domains I and III are close enough for EGF to contact both at the same time. In ERBB3, in the region of the connection between domains I and II, domain II is twisted 30° relative to the configuration of EGFR and ERBB2. As a result the comparable configuration in ERBB3 is wider, suggesting that additional events may be needed for ligand action, or that two molecules of ligand could bind simultaneously.[48,50,51] (4) The latter interpretation is supported by the observation that constitutively locked ERBB3 bound ligand as well as did the extended conformation.[52] (5) ERBB3 does not form stable, ligand-bound homo-dimers,[53] in contrast to EGFR. Part of the reason for this may be amino acid changes in loops adjacent to domain II dimerization arms; disulfide-bonded module 6 is utilized in EGFR dimerization,[54] whereas for ERBB2/ERBB3 heterodimer formation module 7 plays the key role.[55] (6) ERBB3 does however form self-oligomers; both the purified extracellular domain of ERBB3 and the full length protein expressed in insect cells underwent self-oligomerization at low concentrations, comparable to those normally seen on cell surfaces.[56] This oligomerization was destabilized and reduced in the presence of NRG ligand. These features contrast with those of EGFR, with homodimerization induced by ligand and ERBB2, which showed no oligomerization of the extracellular domain. Inability of NRG to cause homodimerization of the extracellular domain of ERBB3 was confirmed with chimeric ERBB3/EGFR and ERBB4/EGFR molecules.[53] By use of a constitutively extended form of ERBB3 it was shown that intermolecular complexes included two different types of interfaces, one involved in oligomerization that is sensitive to NRG disruption, and another for dimer formation that is not affected by NRG.[52] It was proposed that self-associated ERBB3 constitutes the catalytically inactive oligomeric state. Binding of the ligand releases the ERBB3 and may stabilize the extended form of the receptor to expose the dimerization interface for interaction with ERBB2.[52] (7) The extracellular domain of ERBB3 retains NRG ligand binding even at acidic endosomal pH (in both the extended and locked conformations), and the genetically engineered constitutively locked conformation even showed a strong association at minimum pH 5.5.[50] This was in contrast to the binding of TGFα or EGF to the EGFR

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extracellular domain, that is much reduced at low pH. A critical pH-sensitive histidine in domain III of EGFR is absent in ERBB3 and ERBB4.

Overall the unique features of the extracellular domain of ERBB3, as currently understood, seem specifically adapted for highly sensitive activation and fine-tuned control of interaction with ERBB2, including multiple ligand effects, first to disrupt inactivating self-oligomerization of the extended conformation, then to induce and stabilize ERBB2 heterodimer formation. It has been proposed that the locked conformation may have a role especially in endosomal signaling.[7,50]

Transmembrane domain—EGFR, ERBB2 and ERBB4 possess two GXXXG consensus sequences in their transmembrane domain which enhance the efficiency of ligand-induced dimerization.[57] ERBB3 is unique in that it presents only one such sequence and is correspondingly less able to homodimerize.[57] This feature may promote heterodimerization.

Cytoplasmic domain—The important functions of the cytoplasmic domains of the ERBB receptors include interaction with other receptor molecules; specific interactions with downstream substrates and modulators; and stimulation of phosphorylation of self and of substrates.

Several features of the ERBB3 cytoplasmic domain should be mentioned. (1) The tyrosine kinase domain of ERBB3 is largely conserved relative to the other ERBBs, even though it is functionally defective[58] and presumably plays a role in protein–protein interactions. In the kinase region of the protein, the consensus sequence for the ATP-binding site, GlyXGlyXXGly at positions 716–721, is conserved. However, relative to the other ERBB receptors, human ERBB3 has several nonconserved regions in the kinase domain, at positions 740 (Ala instead of conserved Cys), 759 (His instead of Glu) and 834 (Asn instead of Asp).[41] It has been suggested that these substitutions contribute to the impaired kinase activity of this protein,[17] resulting in a 100-fold reduction in capacity for autophosphorylation and substrate phosphorylation, but site-directed mutation at 759 and 834 to glutamate and aspartate, respectively, did not restore kinase activity.[59] Rat Erbb3 presents the consensus Asp at the site equivalent to human 834, and when this was mutated to Asn, interaction of the rat Erbb3 with its targets Ptpn11 (Syp) and PI3K were greatly increased in a yeast two-hybrid assay.[60] Furthermore, mutations at the sites equivalent to 740 or 759 gave additional enhancement of these interactions. These results indicate functional significance for the ERBB3-specific changes at these sites with regard to downstream signaling.

An ongoing question has been how ERBB3, with its impaired kinase activity, could transphosphorylate ligandless ERBB2 within a simple ERBB2/ERBB3 heterodimer. Possibilities include sufficiency of the very low kinase activity of ERBB3 or involvement of another kinase recruited by ERBB3. Studies with transfected mutants of ERBB3 and ERBB2 appeared to rule out participation of ERBB3 as a kinase and of another cytoplasmic kinase.[61] It is most likely that allosteric interactions between lobes of the kinase regions of ERBB2 and ERBB3 result in activation of ERBB2.[8] (2) The C-terminal domain of ERBB3 is 30 to 50% longer than the comparable regions of EGFR and ERBB2. (3) There are three sequential amino-acid residues in this region, Leu^{957}, Val^{958} and Ile^{959}, that are required for transactivation of ERBB2 and that are conserved among EGFR, ERBB3 and ERBB4, whereas ERBB2 differs at the position equivalent to site 957.[61] (4) The same amino-acid substitutions at positions 931, 934 and 966 of ERBB3, relative to the other ERBBs, in three diverse species (human, rat and pufferfish) indicate potential functional significance.[41] (5) The carboxy terminal region of ERBB3 includes 13 tyrosines and the sequence Tyr-Glu-Tyr-Met is repeated three times. (6) ERBB3 has a nuclear localization signal near the C-terminus of the protein.

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Turnover, localization and trafficking

Turnover—The biosynthesis time for ERBB3 is estimated to be ½h, with a half life of 2–3 h. Inactivation and turnover involves dephosphorylation, proteolysis and re-cycling from endosomal compartments. A computational model based on data for H292 human lung carcinoma cells led to the conclusion that dephosphorylation of ERBB3 as well as of EGFR and ERBB2 occurs mainly in the intracellular, endosomal compartment, rather than at the cell membrane.[62]

Unlike the EGF-activated EGFR, which undergoes lysosomal routing and ligand-mediated degradation, ERBB3 is not subject to ligand-induced proteolysis but rather is processed by slow endocytosis with relatively late ligand degradation, followed by rapid recycling.[63–65] This difference is a function of the cytoplasmic domain, as fusion of the C-terminal region of ERBB3 to EGFR resulted in a recycled rather than degraded chimeric molecule.[66] ERBB2/ERBB3 heterodimers similarly undergo slow endocytosis. Signaling may continue within the endosome, dependent on ligand binding; NRG binding to ERBB3 is stable at endosomal pH.[45,50] The nature of the ligand may influence receptor stability. Whereas most EGFR ligands led to rapid degradation of this receptor, betacellulin bound-EGFR was stable at endosomal pH.[50]

Degradation of ERBB3 occurs in proteasomes and is regulated by the recently identified E3 ubiquitin ligase, neuregulin receptor degradation protein (NRDP1), a ring finger protein also known as RNF41 or Flrf. It was discovered as an ERBB3-interacting protein by yeast two-hybrid analyses.[67,68] NRDP1 associates with ERBB3 and stimulates its ubiquitination and rapid degradation by proteasomes in a ligand-independent manner, thus regulating steady-state levels. The C-terminal domain of NRDP1 associates with the cytoplasmic tail of ERBB3. The N-terminal RING finger promotes ERBB3 ubiquitination and degradation. Coexpression experiments indicated that NRDP-1 specifically interacts with ERBB3 and ERBB4 and not with EGFR or ERBB2. As observed in cotransfection experiments, NRDP1 redistributed ERBB3 from the cell surface and was colocalized in the intracellular compartments, particularly perinuclear regions.[68] NRDP1 itself is highly labile and undergoes self-ubiquitination and is degraded through a proteo-some-mediated pathway.[69] NRDP1 correlated negatively with ERBB3 levels in primary breast cancers from both humans and mice and overexpression of NRDP1 led to reduced ERBB3 levels and inhibition of mammary cancer cell growth and motility, whereas reduction in NRDP1 had the opposite effects.[70]

ERBB3 may also be negatively regulated by the leucinerich repeat protein LRIG1, which colocalizes with ERBB receptors and apparently enhances ubiquitination.[71]

Most recently, ERBB3 stability has been found to be regulated also by the NRG isoform that activates it.[72] This study utilized recombinant, nonglycosylated NRG1-β1, the subtype which binds preferentially to ERBB3, with or without N-terminal domains in addition to the EGF-like domain. The presence of N-terminal domains stimulated ERBB3 degradation in MCF7 mammary carcinoma cells. This effect was sequence-independent, as substitution of other peptides of equal size did not abrogate it and was correlated with ability of the full-sized NRG1 to disrupt higher order oligomers of ERBB3.

Nuclear localization—In addition to cell membrane and cytoplasmic localization, all ERBB family members have been observed in cell nuclei.[73–75] This localization has been most extensively studied for EGFR and has been proposed to involve routing from endosomes or direct extraction from cell membrane. Suggested functions for EGFR in the nucleus have included action as a transcription factor, a chromatin re-modeling agent, an agent in DNA repair and/or a signal transducer by means of its tyrosine kinase activity. Nuclear localization of EGFR has typically been described as a response to cell stress or as a concomitant of cell

proliferation, as in hepatic regeneration and in various cancers. Only a few studies of nuclear localization of ERBB3 have been reported, but these indicated potentially interesting functions in this compartment. In immortalized human breast cells and breast cancer cell lines, full-length ERBB3 showed prominent nuclear localization with several antibodies and techniques.[76] An active nuclear localization signal was confirmed in the C-terminal region of the ERBB3. Amounts in the nucleus were increased by treatment with a nuclear export inhibitor. However, neither NRG nor ERBB2 was present in the nuclei of these cells. When immortalized cells became differentiated and polarized as a result of growth on a permeable membrane, nuclear ERBB3 was localized primarily in nucleoli. Exposure to NRG resulted in shift of the ERBB3 from nucleoli, to nucleoplasm, to cytoplasm. These results suggest a role for ERBB3 in regulation of RNA synthesis during growth arrest, and downregulation of this role by cytoplasmic sequestration with NRG during proliferation.

In a series of Japanese lung cancers, ERBB3 was detected in the nucleus of 57%, associated with significantly higher levels of *ERBB3* mRNA.[77] In a transformed cell line from peripheral mouse lung peripheral epithelium, ERBB3 was detected in the nucleus and became enriched in the nucleoli of serum-starved cells.[78] As for the breast cancer cells, treatment with NRG resulted in movement of the ERBBs out of the nucleus into the cytoplasm.

ERBB3 has also been detected in nuclei of prostate cancers and cancer cell lines.[79] Its nuclear levels were low or absent in nonmalignant tissue and higher in hormone refractory compared with hormone-sensitive cancers and so were apparently correlated with tumor progression. In contrast, nuclear ERBB3 was higher in prostate cancer cell lines that were androgen responsive. Treatment of the cells with NRG resulted in tyrosine phosphorylation of ERBB3 in the cytoplasm, but not the nucleus, consistent with lack of ERBB2/ERBB3 heterodimer in nuclei of breast cancer cells.[76] ERBB3 was also noted in nuclei of Schwann cells.[80]

In sum, it seems likely that nuclear localization of ERBB3 has major functional importance in health and disease and is a compelling subject for future study.

ERBB3 interacting proteins: activation, signaling and regulation

Activation (Table 2)

Ligand-dependent activation—The primary ligands for ERBB3 are the members of the NRG family, a large group of isoforms encoded by four genes, with an EGF-like C-terminal portion and a variable N-terminal region. Several recent comprehensive reviews of NRGs are available.[81–83] Alpha and β isoforms utilize different exons for the EGF-like domain. The relative effects of the NRGs on ERBBs appear to relate in part to the cells under study and the type of assay. ERBB3 affinities for the EGF-like domains have been measured in a direct binding assay.[84] NRG1β bound with much greater affinity than NRG1α. When ERBB2 and ERBB3 were present together, relatively weak binding was also detected for NRG2β and epiregulin α. NRG2α and 3α, betacellulin α, heparin-binding EGF, EGF and TGFα were negative.

Consistent with these results, NRG1β had a much stronger stimulatory effect on DNA synthesis in NIH3T3 cells, compared with NRG1α.[85] Similarly in T47D mammary and OVCAR3 ovary cancer cells NRG1β caused greater activation of ERBB3, ERBB4 and ERBB2 and more persistent ERK2 activation than did NRG1α.[86] In MDA-MB-453 breast carcinoma cells, NRG1β and NRG2β caused equivalent levels of ERBB2 and ERBB3 phosphorylation, but NRG1β led to increased or prolonged signaling through AKT, ERK, PKC, RSK, S6K, MYC, JUN and CREB compared with NRG2β, and also generated a different gene expression profile.[87] This was associated with lower recruitment of ERBB2 signaling partners after NRG2β compared with NRG1β.[88]

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Nevertheless in some breast cancer cell lines NRG2 is active. Cell lines MDA-MD-453 and T47D were stimulated to grow by two NRG2 isoforms.[89] NRG3 was expressed in breast cancer cell lines, and the EGF-like domain of NRG3, as a recombinant protein, activated ERBB2/ERBB3 in breast cancer cells and altered their growth,[90] although it had little affinity in a direct-binding assay.[84] NRG4 appears to be specific for ERBB4.[91]

In the context of the ERBB family members expressed individually or in pairs in myeloid cells, NRG1α and β and NRG2α and β had no effect on ERRB3 expressed alone but were equally effective in activating it in the presence of ERBB2; similar though lesser effects were seen with ERBB3+ERBB4.[92] When ERBB3 was expressed with EGFR, only NRG1β and NRG2α had effects.[92]

Altogether the effects of activation of ERBB3 by NRGs probably depends on the amounts and ratios of the NRG isoforms present, their status as secreted, paracrine or autocrine factors and the relative amounts of other ERBBs. An additional complexity has recently been added with the finding that in gefitinib-treated breast cancer cells NRG changed in both amount and in nuclear localization, in opposite directions depending on the gefitinib responsiveness of the cells.[93]

Ligand-specific cellular effects have also been described for ERBB4 and related to phosphorylation of specific tyrosine residues.[94] Several possible mechanisms were postulated, including recruitment of cytosolic kinases or phosphatases, extracellular regulators or signal-regulatory proteins and differential receptor aggregation or conformation change.

Effects of NRG1β on membrane localization of ERBB3 have recently been studied by immunoelectron microscopy. ERBB3 expressed in stably transfected CHO cells formed clusters in the cell membrane.[95] The sizes of these clusters were similar with high or low receptor expression, and increased when NRG was added. In SKBR3 mammary carcinoma cells, NRG also led to large increases in ERBB3 clusters and marked coclustering of ERBB3 with the p85 subunit of PI3K, but not with ERBB2 or EGFR.

Other ERBB family ligands may also have effects under some circumstances. In a direct binding assay, EGF and betacellulin did not activate ERBB3 when expressed alone, but these ligands did have low-affinity activity when ERBB3 was expressed in cells with ERBB2; TGFα, amphiregulin and heparin-binding EGF remained in-effective.[84] Similar results were obtained with MDA-MB134 and MDA-MB453 mammary carcinoma cells after prolonged exposure to EGF or betacellulin.[96,97] Somewhat different results were obtained with T47D mammary tumor cells, where betacellulin and heparin-binding EGF but not EGF activated ERBB3.[98] Finally, in a wound repair model, transfected ERBB3 enhanced various wound healing parameters more effectively when combined with epiregulin or HB-EGF, than with EGF or NRG.[99]

Physical interaction with other ERBB family members—Since ERBB3 has only minimal intrinsic kinase activity, its phosphorylation after NRG activation is dependent on physical association with other ERBB family members, to provide highly potent heterodimers.[100] Phosphorylation may also occur without NRG, via EGFR activation by its specific ligands.[101] Complexation of ERBB3 with EGFR is a prominent phenomenon in some models.[101–103] In several different types of transfected cells, the expression of the EGFR was sufficient to allow activation of ERBB3 in response to EGF.[104–106] ERBB3 was a highly receptive substrate for EGFR tyrosine kinase activity.[107] Activity of purified EGFR toward tyrosine-containing peptides from the C-terminal region of ERBB3 was measured *in vitro*.[108] Activity was high toward the peptides with two tyr (1197/1199, 1222/1224 and 1260/1262) as well as toward that with tyr1159. EGF increased EGFR catalytic activity toward ERBB3 phosphorylation

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sites, but affinity was not changed. Certain sites had higher specificity constants than any EGFR sequence. However the tyr1289 in ERBB3, important as a PI3K binding site, had relatively low activity with EGFR. ERBB3 also has the potential to activate EGFR: in several models involving high expression of transduced ERBBs, NRG-stimulated ERBB3 activated EGFR.[104,109] However, in five human mammary and ovarian carcinoma cell lines expressing both receptors no EGFR tyrosine phosphorylation occurred after NRG treatment.[86]

Interaction of ERBB3 with the ligand-less ERBB2 results in a complex with enhanced affinity for NRG and increased ERBB3 phosphorylation,[106,110,111] and these receptors, as noted below, contribute synergistically to cell transformation and to malignant properties of cancer cells. ERBB2 is more likely to heterodimerize with ERBB3 than to homodimerize.[112] NRG-induced formation of the ERBB2/ERBB3 complex resulted in conversion of ERBB2 from an inhibited to an active protein conformation.[113]

ERBB2 participates in communications between ERBB3 and the EGFR: stimulation of PC12 cells with either EGF or NRG led to formation of complexes containing EGFR, ERBB2 and ERBB3; it was proposed that primary dimers of EGFR/ERBB2 after EGF and of ERBB3/ERBB2 after NRG underwent dissociation, and secondary dimers formed of ERBB2/ERBB3 or ERBB2/EGFR, respectively.[114] A site-specific mutagenesis study identified ERBB2 sites L295 and H296 as critical to ERBB2/ERBB3 heterodimerization in response to NRG.[55] A monoclonal antibody to the EGFR stimulated the growth of NSCLC line PC-14 by enhancing ERBB2/ERBB3 heterodimerization, possibly by blocking hetero-dimerization of EGFR with ERBB2 or ERBB3.[115]

Herstatin, a naturally occurring ERBB2 inhibitor, prevented transactivation of ERBB3 in response to NRG in the context of CHO cells transfections.[116] However, herceptin, a monoclonal antibody to ERBB2 which targets ERBB2/EGFR heterodimers, had no effect on ERBB2/ERBB3; ERBB3/EGFR heterodimers were unstable in this engineered cell expression system.[117]

ERBB3/ERBB4 complexes have also been reported[78] and can stimulate cell division.[92]

ERBB3 phosphorylation by other kinases—Other kinases may also phosphorylate ERBB3 under some circumstances. Also in mammary cancer cells, expression of a kinase-dead EGFR mutant blocked activation of EGFR, ERBB2 and ERBB4, but basal tyrosine phosphorylation of ERBB3 was enhanced and c-SRC was implicated by specific inhibitor studies.[118] C-SRC binds a phosphotyrosine site in ERBB3 (see Table 3). Tyrosine phosphorylation of ERBB2 and ERBB3 and formation of their heterocomplex, as well as downstream signaling, was enhanced by expression of c-SRC in both fibroblasts and breast cancer cells.[119] A SRC-family kinase inhibitor reduced phosphorylation of ERBB3 at Y1289, the binding site for SRC, especially in EGFR-dependent NSCLC cell lines HCC827 and H3255.[120]

Recently lung cancer cells with *EGFR* mutations but with resistance to gefitinib therapy were found to have amplification of the gene for the MET receptor, a transmembrane tyrosine kinase that is activated by hepatocyte growth factor.[121,122] Physical complexes of MET with ERBB3 and PI3K were demonstrated. The downstream activation of PI3K and AKT via the MET/ERBB3 interaction accounted for the acquired gefitinib resistance.

In myotubes, Nrg caused activation of cyclin-dependent kinase 5 (Cdk5), in addition to the expected tyrosine phosphorylation of Erbb3 and inhibition of Cdk5 by several means led to reduced tyrosine phosphoryation of Erbb3 in response to Nrg.[123] Furthermore Cdk5 and Erbb3 coimmunoprecipitated, and Cdk5 caused ser/thr phosphorylation on immunoprecipitated

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Erbb3. These results for muscle were confirmed in *Cdk5*$^{-/-}$ knockout mice.[124] In a further study, Cdk5 immunoprecipitated from brain extracts was demonstrated to phosphorylate Thr 871 and Ser 1120 in rat Erbb3 *in vitro*;[125] the consensus sequence of RSRSRSPRPR surrounding Ser 1120 is novel. Physical association of Cdk5 and Erbb3 was confirmed in rat cortical neurons. Cdk5 also phosphorylated Erbb2. In contrast to the situation in muscle, Cdk5$^{-/-}$ neurons showed reduced Erbb3 ser/thr phosphorylation and lowered PI3K activity, without a reduction in Erbb3 tyr phosphorylation. This suggests that, at least in neurons, ser/thr phosphorylation may directly regulate Erbb3 function.

The breast cancer-associated tyrosine kinase BRK (also known as protein tyrosine kinase 6, PTK6), which is tyrosine-phosphorylated in mammary epithelial cells upon EGF treatment, further caused an increase in tyrosine phosphorylation of ERBB3 in response to EGF. It also coimmunoprecipitated with ERBB3 when both were overexpressed in human embryonic kidney cells.[126] Direct phosphorylation of ERBB3 by BRK was postulated but has not yet been directly demonstrated.

Regulation of ERBB3 activation by feedback effects from AKT—Qualitatively new insight into ERBB3 regulation developed from a study of escape of breast cancer cells from suppression by tyrosine kinase inhibitors.[127] After prolonged exposure of BT474 or SKBR3 mammary cancer cells to gefitinib, erlotinib or AG825, the initially suppressed pERBB3 and pAKT levels recovered, even while pEGFR and pERBB2 remained inhibited. This effect correlated with increased levels of ERBB3 in the cell membranes and was dependent in part on increases in intracellular peroxides. Effects of a PI3K inhibitor and of constitutively active AKT implicated negative feedback from AKT in compensatory upregulation of pERBB3 levels.

Other modes of ERBB3 activation—In the MCF10A nontransformed mammary cell line, NRG activated ERBB3 without apparent involvement of the other ERBBs.[128] ERBB3 may be transactivated by cellular stress and cytokines, including tumor necrosis factor α and interferon α.[129,130] Mechanisms have been further elucidated for multiple myeloma cells, and Janus tyrosine kinases TYK2 and JAK1 have been implicated, though neither demonstrated physical association with ERBB3.[131]

Proteins binding with phosphotyrosines in ERBB3′s cytoplasmic domain

Sequence analysis of ERBB3 indicated putative binding sites for SHC, GRB7, GRB2, SRC and the p85 regulatory subunit of PI3K.[59] These have been empirically confirmed and other proteins interacting with the C-terminal cytoplasmic domain of ERBB3 discovered, using several types of arrays and yeast-two hybrid assays. Schulze *et al*.[132] used an array method to pull down proteins in lysates of HeLa cells by each of the phosphotyrosine-containing peptides in the ERBB family members. For ERBB3, binding of PI3K p85 to six sites was confirmed, GRB2 associated with two sites and SHC and SRC were each pulled out by one site. ERBB3, uniquely among the family, has three pairs of tyrosine residues separated by a single glutamic acid residue (YEY motif). For all three, p85 bound to the first phosphorylated residue, and for two of these motifs, GRB2 bound to the second. Doubly-phosphorylated YEY motifs did not have any unique properties in these assays.

A remarkable accomplishment was recently reported by Jones *et al*.:[133] all of the SRC homology 2 (SH2) and phosphotyrosine-binding (PTB) domains encoded in the human genome were measured for binding to each of the phosphopeptides from the ERBB receptors. This included 106 SH2 domains and 41 PTB domains, and for ERBB3, 10 phosphotyrosine-containing peptides and their non-phosphorylated counterparts. Binding to ERBB3 was detectable for 46 of the tested domains. The peptides interacting with pY residues in ERBB3

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with high or moderate affinity (Kds of <1000 nM) in this study are listed in Table 3, including four that had been previously reported, PI3KR1/2/3 (PI3Kα), GRB7, SHC1 and PTK6. GRB2 and PTPN11, described as binding in other studies, did not interact with ERBB3 in this test system. Phosphotyrosine sites Y1276 and Y1289 showed many high-affinity interactions, whereas other sites were markedly more selective. Overall the ERBB3 sites averaged 8.8 domains with high-affinity interaction. ERBB2 by contrast had many promiscuous sites. The binding profile of ERBB3 changed less with varying concentrations of PTB and SH2 domains than did those of EGFR and ERBB2.

PI3K regulatory subunit p85 and AKT—The most fully studied target of ERBB3 is the p85 regulatory subunit of PI3K (PI3KR), with potential for strong mitogenic signals.[66] *In vitro* phosphorylation of ERBB3 by EGFR resulted in strong association with p85 and activation of PI3K.[105] A proliferative response of NIH3T3 cells to NRG, dependent on expression of ERBB3, involved association of PI3K with ERBB3.[134] Early studies indicated that a prominent association between ERBB3 and p85 is a unique feature of ERBB3,[135] and this was confirmed by the assays of Jones *et al.*,[133] which found that both SH2 groups in each of the three PI3KR isoforms bound with moderate or high affinity at multiple sites in ERBB3, as previously reported.[104] However, EGFR and ERBB2 (but not ERBB4) did bind to p85 at a limited number of sites.[133] Each of the p85 sites contributes to ERBB3 signaling, as demonstrated by their one-at-a-time mutation and restoration, and cooperation among p85-binding sites was observed.[136] These interactions involve the N-terminal SH2 domain of p85, with the two phosphotyrosine-binding sites in this domain each interacting preferentially with certain phosphotyrosyl peptides from ERBB3.[137] For the three pairs of tyrosine residues separated by a glutamic acid, the first Tyr in each case binds p85.[132] Doubly phosphorylated tripeptides also bound p85, with the two phosphotyrosine-binding sites in p85 possibly each engaging a different but nearby phosphotyrosine.[137] Because of the high number of binding sites, ERBB3 is viewed as a possible scaffold protein for PI3KR.

One of the best characterized targets of PI3K is the kinase AKT. ERBB3/PI3K/AKT-induced survival and proliferation pathways have been implicated in the malignancy of breast, ovarian, colon, gastric and lung cancer cells. Various approaches using ERBB3 mutants,[136,138] immunoprecipitations with antibody against ERBB3[139,140] or NRG,[141] ERBB3 antisense,[139] ERBB3 small interfering RNA (siRNA),[142] and use of the designer ERBB3 transcription inhibitor E3 in a variety of cells[143] have established the importance of this pathway.

GRB7/GRB2—GRB7 was notable for its uniquely high-affinity binding with pY1197 in ERBB3 (Table 3). GRB7 is an adapter molecule and has a role in integrin signaling and cell migration in various cell types. It can be overexpressed in breast, esophageal and gastric cancers and has been proposed for therapeutic targeting.[144] In human breast cancer cell lines co-immunoprecipitation of GRB7 and ERBB3 was detected upon NRG stimulation.[145] The association was direct and mediated by the GRB7 SH2 domain; this study also indicated pY1197 and pY1260 as the major and minor sites of GRB7 interaction in ERBB3. Although these recognition sequences represent GRB2-binding sites, ERBB3 preferentially bound to GRB7. This was also observed with EGFR/ERBB3 chimeras expressed in NIH3T3 fibroblasts.[59] However, in HeLa cells interaction with ERBB3 was demonstrated for GRB2, whereas GRB7 was not present in these cells.[132]

SHC—The adapter SHC is unique among the well-studied ERBB3 targets in that binding preferentially involves the amino-terminal PTB domain, rather than the carboxy-terminal SH2 domain.[59,146] This was confirmed by Jones *et al.*,[133] with pY1328 identified as the site of interaction. Signal transduction to SHC from ERBB3 occurs after NRG or EGF exposure in NIH 3T3 cells.[106] Site-directed mutagenesis studies in these cells indicated that NRG stimulation of mitogenesis involved both MAPK and PI3K pathways from ERBB3, with the

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ERBB3 SHC-binding site at rat Tyr1325 essential for the MAPK pathway stimulation.[147,148] In MDA-MB-468 human mammary cancer cells, NRG1β preferentially stimulated ERBB3, resulting in recruitment of SHC.[88] ERBB3/SHC interactions were involved in NRG stimulation of transcription of the acetylcholine receptor gene in muscle cells.[149]

PTK6—Protein tyrosine kinase 6 (PTK6, BRK), when highly expressed in mammary epithelial cells, resulted in enhanced phosphorylation of ERBB3 and downstream activation of AKT.[125] Its SH2 domain bound only at pY1276 (Table 3) and did not have affinity for the other ERBB proteins.

c-SRC—Physical association occurred between c-SRC and ERBB3 in mammary cancer cells, and when both were stably expressed in CHO cells; however, neither tyrosine kinase activity of c-SRC nor tyrosine phosphorylation of ERBB3 was required for this complex.[118] In the presence of kinase-dead mutants of EGFR, ERBB2 and ERBB4, ERBB3 passed an anti-apoptotic signal through c-SRC.[118] The SH2 domain of SRC bound only at pY1289 (Table 3) and bound only to ERBB3 in this assay.

PLCG1—Phospholipase γ1, a signal transducer, has generally been thought to be a substrate specific to the EGFR,[66,135] but an association between ERBB3 and PLCG1 was observed in irradiated A431 carcinoma cells.[150] Its interactions with ERBB3 were limited to pY1276 and pY1289 (Table 3).

Newly-identified proteins binding ERBB3 phosphotyrosines—The assay of Jones *et al*.[133] revealed a number of hitherto unsuspected, interesting proteins whose SH2-domains had affinity for ERBB3 phosphotyrosines. ABL2, a cytoplasmic tyrosine kinase, bound at seven sites and was the only candidate with high affinity for pY868. ABL1 by contrast had affinity only at pY1289. RASA1N, a Ras regulatory protein, bound at five sites and was one of three with affinity for pY868. Ras regulatory protein was present in multimeric complexes with ERBB proteins after NRG stimulation of breast cancer cells.[151] The third protein interacting at pY868 was SYK, a cytoplasmic tyrosine kinase with central signaling roles in hematopoietic cells and a tumor suppressive function in mammary cells.[152] Several adapter proteins, CRK, NCK1 and NCK2 and the ras/jun activator CRKL, bound with high affinity only to pY1276 of ERBB3. The signaling protein JAK2 was also bound at pY1276. NRG1 activated JAK3, but not JAK1 or JAK2, in lung epithelial cells.[153] JAK3 also bound at pY1276 but with lower affinity (Kd 1355 nM).

VAV1, an oncogene and a member of the DBL family of Rho guanine nucleotide exchange factors, bound especially at pY1289, while VAV2, also an oncogene and a SRC effector, had high affinity for pY1276. VAV3, a guanine nucleotide exchange factor for both RHO and RAC, also bound at pY1276 but with lower affinity (Kd 1539 nM).

TENC1, which may be a focal adhesion molecule, was bound mainly at pY1222. Several proteins bound with moderate or high affinity only at pY1289: the oncogene homolog LYN; TENS1, which has a role in disassembly of EGF-related signaling complexes at focal adhesions; FER, a nonmembrane receptor tyrosine kinase that regulates intercellular adhesions; ITK, an intracellular tyrosine kinase; and DAPP1, a protein phosphatase.

An interesting possibility is that SH2 domains which bind with moderate or high affinity at only one site, as is the case for CRK, NCK1, NCK2, CRKL, JAK2, PTK6 and VAV2 at pY1276 and for SRC, VAV1, LYN, TENS1, FER, ITK and DAPP1 at pY1289, compete for binding at these sites, so that their relative concentrations determine the nature of the signal generated.

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Other intracellular proteins interacting with ERBB3 EBP1—A particularly interesting binding partner for ERBB3, discovered in a yeast two-hybrid assay, is ERBB3-binding protein 1(EBP1).[154] EBP1 is encoded by human gene PA2G4 (human homolog of the mouse p38-2G4 protein, a cell cycle-regulated DNA-binding protein), and is widely expressed.[155] It interacts with the first fifteen amino acids of the juxtamembrane domain of unphosphorylated ERBB3. A functional story has emerged for EBP1 in human breast and prostate cancer cell lines, where high expression of EBP1 leads to reduced cell growth and increased differentiation.[156,157] In these cells, binding of EBP1 to ERBB3 is dependent on constitutive phosphorylation by PKC.[158] Upon NRG stimulation, EBP1 is phosphorylated, independent of PKC, dissociates from ERBB3, and translocates to the nucleus.[154,155] Nuclear EBP1 interacts directly with the cell cycle regulator pRB, resulting in inhibition of transcription of E2F-regulated genes, including cyclin E, by a mechanism including recruitment of Sin3A and histone deacetylase.[157,159–162] EBP1 contains an LXXLL motif mediating interactions with nuclear hormone receptors and binds androgen receptor, resulting in inhibition of activation of androgen-responsive gene promoters.[157,163,164] Transcriptional effects of EBP1 are dependent on phosphorylation at serine 363.[165] Thus, in both breast and prostate cancer cells, EBP1 is potentially a critical effector for ERBB3 signaling.

EBP1 may have even wider and more complicated roles, as shown by recent results with other cell types. Nucleolar localization was noted for EBP1 in HeLa cells, 3T3 fibroblasts and mouse mammary epithelial cells,[166,167] as part of ribonucleoprotein complexes and in association with different rRNA species via its dsRNA-binding domain and sigma70-like domain. Furthermore, in the cytoplasm EBP1 associates with mature ribosomes and potentially influences protein translation via inhibition of phosphorylation of eukaryotic initiation factor 2α.[167] Most recently, in PC12 pheochromocytoma cells, two isoforms of EBP1 were discovered, with differing properties with regard to ERBB3-binding, intracellular localization and effects on cell survival and differentiation.[168]

The crystal structures of human EBP-1[169] and of murine Ebp-1[170] have recently been reported, and a requirement demonstrated for the C-terminal region in RNA binding.[170]

BMS/ETK—The nonreceptor tyrosine kinase BMS/ETK was activated by NRG1β and formed a complex with ERBB3 in prostate cancer cells.[171] Activation also required PI3K activity through a membrane-targeting effect of phosphatidylinositol-3-phosphate. Thus ERBB3 appeared to be involved both directly and indirectly in activation of this growth-stimulatory signaling molecule.

ERBB3 interacting factors in yeast two-hybrid assays—Three additional ERBB3-interacting proteins were identified using the split-ubiquitin membrane yeast two hybrid system, wherein a human brain library was screened with ERBB3 as bait.[172] RGS4 (Regulator of G protein-signaling family member) was one of the interacting proteins. This interaction was further confirmed by demonstration that transiently expressed ERBB3 and RGS4 formed coimmunoprecipitation complexes in human HEK293T cells. This screen also revealed interactions between ERBB3 and Early Growth Response Protein 1 (EGR1), a zinc finger transcription factor important for neurite outgrowth, wound repair growth control and apoptosis; and ZNF207, a hypothetical zinc finger transcription factor. Possibly these interactions are important in the nuclear functioning of ERBB3 (above). A yeast two-hybrid assay was also used to discover an interaction between the angiotensin II receptor and the ATP-binding domain of ERBB3.[173] The functional significances of these interactions remain to be demonstrated.

The human homolog of the mouse transplantation antigen P198, designated p23, was found to interact with the cytoplasmic domain (juxtamembrane region) of ERBB3 in a yeast two-hybrid

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assay.[174] Transfection of p23 into ERBB3 overexpressing mammary cancer cells resulted in decreased growth and induction of differentiation.

Some of the interesting proteins interacting physically with ERBB3 in the cytoplasm are summarized in Table 4.

ERBB3 in normal and neoplastic tissues

Cell transformation by ERBB3

In view of the demonstrated effects of ERBB3 on cell division and survival, it is not surprising that it can contribute as an oncogene to cell transformation and tumorigenesis, particularly when acting in concert with ERBB2. Transfection of ERBB3 into NIH3T3 fibroblast cells resulted in a low level of colony growth in soft agar,[17] but ERBB3 coexpressed in these cells with ERBB2 greatly enhanced the degree of transformation seen compared with ERBB2 alone.[175] ERBB2 activity and action of ERBB3 in concert with ERBB2 as a heterodimer were required for this transformation, and phosphorylation of ERBB3 and activation of PI3K were associated. In another study with transfected 3T3 cells, ERBB3 was again not transforming by itself, and transformation when combined with ERBB2 required that NRG be expressed as well.[176] Similarly transformation of 3T3 cells by NRG required coexpression of ERBB3 and ERBB2 or ERBB4, with the former being the more effective.[86] 3T3 cells overexpressing only ERBB3 formed small tumors as nude mouse xenografts, but only after a long latency suggestive of need for additional events.[111] Tumors resulting from combined expression of ERBB2/ERBB3 or EGFR/ERBB2 made high levels of vascular endothelial growth factor, compared with other receptor combinations.

In an extensive investigation which included microarray analysis of gene expression, all combinations of the ERBB receptors were expressed in 3T3 cells.[177] ERBB3 alone or in combination with EGFR was not tumorigenic. ERBB3 in combination with ERBB2 transformed the cells as expected, and yielded xenograft tumors that grew more aggressively than observed with any other ERBB combinations. Tumors were also induced with cells expressing ERBB3 and 4; these had a slow growth rate. Each cell line expressing single or double receptors had a unique pattern of gene expression. Especially notable was high expression of the genes for insulin-like growth factor 2 and insulin-like growth factor-binding protein 5 in the aggressive tumors induced by ERBB2 plus ERBB3.

NIH3T3 cells were also utilized for a study of differential effects of transfected ERBB3 vs EGFR on gene expression, as analyzed by representational difference analysis. Expression of *dlk*, a gene for a transmembrane protein with EGF-like repeats in the extracellular domain, was upregulated by ERBB3 but not EGFR.[178]

Mammary gland

ERBB3 expression in normal mammary gland and cells—Erbb3 levels were low in embryonic mammary tissue and increased during postnatal maturation, with evidence of activation via phosphorylation in mammary tissue during mid to late pregnancy in mice and high expression in both mammary ductal epithelial cells and stroma in pregnant rats.[179,180] ERBB3 was downregulated in functionally differentiated mammary epithelial cells.[179] In two nontransformed human mammary epithelial cell lines, H16N-2 and MCF-10A, NRG1β was strongly mitogenic and activated PI3K through ERBB2/ERBB3 heterodimer formation;[181] NRG1α was less effective. A significant, though weak, activation of PI3K was also observed after EGF stimulation and formation of an EGFR/ERBB2 heterodimer. Others have also noted ERBB3 expression in MCF10A cells.[182,183] However, in other nontransformed immortalized mammary cell lines, expression of ERBB3 was very low or absent: AB548,[17] HBL100[35,184–187] MTSV1.7[35] and MRSV-2.1 and −2.4.[184] It seems that ERBB3 has regulatable

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expression in nontransformed mammary epithelial cells, and that this program may or may not be activated during establishment of immortalized cell lines.

Mammary tumors in transgenic mice—The great majority of experiments addressing the cancer-related effects of ERBB3 have been carried out in the context of mammary cancer. Contributions of ERBB3 in mammary cancer have been appreciated since the discovery of the gene in 1989: overexpression of *ERBB3* mRNA in some mammary tumor cell lines was reported in the same publication.[17] In transgenic mice, targeting NRG to the mammary gland led to appearance of carcinomas, in which Erbb3 but not Erbb2 or Erbb4 was activated by phosphorylation.[188] The long time course required for tumor appearance (12 months) suggested that chronic activation of Erbb3 was synergistic with or permissive of other transforming events. In mutant ERBB2-driven mammary tumors in transgenic mice, Erbb3 was specifically and markedly increased in amount and constitutively phosphorylated; enhanced Erbb3 protein translation or stability, rather than transcription, was implicated.[189] Tumor cells derived from mouse mammary cancers driven by transgenic rat wildtype Erbb2 also presented high Erbb3 levels, Erbb2/Erbb3 heterodimers, and down-stream activation of PI3K and MAPK pathways by NRG.[190]

Primary breast cancer in humans—For primary breast cancer in humans, increased ERBB3 expression relative to normal is common. These expression increases are not related to increased copy number.[184] In what appears to be the only direct study of ERBB3 protein, 2D-PAGE analysis of four normal and four malignant breast cancer samples revealed the presence of ERBB3 only in the malignant tissue.[191] Immunohistochemical approaches find ERBB3 protein to be detectable in 50–70% of human breast cancers,[192–194] with higher expression of ERBB3 in human breast cancers vs normal tissues in 18–29% of cases.[184,192,195]

ERBB3 mRNAs evaluated by real-time PCR showed a 100-fold variation, and increased expression relative to normal in 46% of breast cancers.[186] In another study, *ERBB3* mRNA had twofold higher expression on average compared with isolated mammary epithelial cells, but there was considerable variability and lack of statistical significance.[196] mRNAs for all four ERBB receptors and their 10 ligands were quantified by real-time PCR for a series of 365 primary breast cancers.[197,198] *ERBB3* mRNA correlated positively with that for *ERBB4* and negatively with *EGFR* mRNA. There was a positive association between *ERBB3* mRNA and mRNAs for estrogen and progesterone receptors and with overall survival, and a negative correlation with histoprognostic grading and with TGFα. Nevertheless, more than 60% of the tumors presented coexpression of high levels of mRNA for *EGFR*, *ERBB3* and *TGFα*, whereas only 39% were positive for *NRG*. Most recently, *ERBB3* was one of a small number of genes found to be overexpressed in malignant vs normal breast tissue by a subtractive hybridization technique and PCR, although the degree of overexpression was not marked.[199]

In one of the studies cited above high *ERBB3* mRNA seemed to be a favorable prognostic indicator. However, in another investigation high *ERBB3* mRNA expression correlated with poor survival.[186] With regard to correlation between mammary cancer prognosis and ERBB3 protein status, high expression as determined by immuno-histochemistry has shown positive associations with metastasis,[184] tumor size and local recurrence,[200] tumor grade[193] and tumor recurrence.[194] Two studies concluded reduced survival associated with ERBB3 protein overexpression,[195,201] whereas several other investigations did not.[200]

There seems to be particular confusion regarding relationships between ERBB3 expression and estrogen receptor (ER). At the level of proteins as determined by immunohistochemistry, ERBB3 and ER did not correlate,[193,200] and a high percentage of ER-negative tumors were strongly positive for ERBB3.[193] Another study seemed to be confirmatory, showing a weak

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inverse relationship between ERBB3 protein and ER.[195] In cultured mammary cancer cells, estrogen treatment suppressed *ERBB3* transcription.[38] On the other hand, for mRNAs, a positive relationship between *ERBB3* and *ER* was noted, along with increased benefit of expression of both with regard to endocrine therapy.[202] Similarly *ERBB3* mRNA expression in 38 mammary cancers was associated with ER positivity and correlated with ERα mRNA as well as with *estrogen-related receptor* α mRNA.[196] It has been suggested[195] that these discrepancies may be explained in part by the presence of soluble, inhibitory sERBB3. The relationship between ERBB3 and ER may be important to understand, since overexpression of ERBB3 predicted relapse during tamoxifen treatment for breast cancer.[203]

At present it seems that simple determination of levels of ERBB3 mRNA or protein in mammary cancers does not lead to sure biological or clinical predictions, probably due to the many other factors that influence its expression, activity, localization and pathway interactions.

ERBB3 signaling in mammary cancer cell lines—Many of the important features of ERBB3 signaling have been discovered in cell lines derived from mammary cancer. In 35% of such lines *ERBB3* transcript was expressed at high levels, relative to a nontransformed mammary cell line.[17] As noted above, amounts of ERBB3 in cultured mammary carcinoma cells may be regulated by estrogen and by integrin. Ethanol, which has been linked epidemiologically with breast cancer risk, resulted in increased levels of ERBBs 2, 3 and 4 in T47D breast carcinoma cells, leading to increased invasiveness in response to NRG.[204]

Human mammary carcinoma cell lines contributed to the demonstration of NRG as a stimulatory ligand for ERBB3.[205] The conclusion that an ERBB2/ERBB3 complex constitutes a high affinity ligand for NRG[110] has been amply confirmed in mammary carcinoma cells, along with mutual phosphorylating transactivation by ERBB2 and ERBB3.[175,176,185,205–216] ERBB3 can also be transactivated by the EGFR in mammary cancer cells.[105,217] Different breast cancer cell lines show considerable variability with regard to relative expression, colocalization, responsiveness and activity of ERBB3 and the other ERBB receptors.[218,219] Complexes of activated ERBB3 with the p85 regulatory subunit of PI3K, along with increased PI3K activity and elevations in pAKT, have also been repeatedly demonstrated in mammary carcinoma cells,[105,175,176,185,213–215,220] due either to constitutive activity or to treatment with NRG or EGF.

The role of ERBB3 was shown in a particularly definitive study by Holbro *et al*.,[143] in which ERBB3 was downregulated with a designer transcription factor in the SK-BR-3 mammary carcinoma cell line with high expression of ERBB2. The cells were blocked in cell cycle G1 phase and presented much reduced pAKT and, downstream, cyclin D3 and pRB. These effects were reversed with an ERBB3 expression vector or with constitutively active AKT.

Several other studies have described cell cycle-related events in mammary carcinoma cells after ERBB2 inhibition, with blockage of ERBB2/ERBB3 heterodimer formation and AKT activation as the likely intermediary: cell cycle arrest with reduced c-myc, cyclin D and CDK1 activity,[211] RB-dependent G1 arrest with downregulation of cyclin D,[213] and increased p27 and reduced cyclin D and colony formation.[214] Recently RAC1 has been identified as an important mitogenic mediator after NRG treatment of mammary cancer cells, with ERBB3, ERBB2 and EGFR all involved.[221]

As noted above, ERBB3 may be activated directly by NRG, or via heterodimer formation with activated EGFR or ERBB4. Most studies have focused on NRG effects. NRG is mitogenic for most mammary carcinoma cell lines,[82,222–225] and can also stimulate motility and invasiveness of these cells.[209,225–227] In ERBB2-over-expressing breast tumor cells, G1 progression after NRG stimulation was associated with ERBB2 transactivation of ERBB3 and stimulation of

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the PI3K pathway.[228] Contributions of ERBB3 and/or the ERBB2/ ERBB3 complex to these phenotypic effects of NRG has been confirmed by use of anti-ERBB2 or anti-ERBB3 antibodies[211,212,229] or of a dominant-negative ERBB3 construct.[230]

The downstream participation of PI3K or pAKT was confirmed in some of these studies by use of pharmacological or dominant-negative inhibitors of PI3K[209] or by constitutively active constructs of the p110 catalytic subunit of PI3K and by pharmacological inhibition of AKT.[213] Additional signal transducing molecules, potentially important for the malignant phenotype, such as JNK, MAPK and p38 MAPK, have been implicated downstream of NRG in mammary cancer cells, along with altered transcription of cancer-related genes such as matrix metalloproteinases, urokinase plasminogen activator, vascular endothelial growth factor, angiogenic factor Cyr61, autocrine motility factor, HIF1α, activating transcription factor 4, GADD153, estrogen and progesterone receptors and BRCA1 (reviewed in[82]).

In tamoxifen-resistant MCF7 mammary carcinoma cells, NRG1 caused heterodimerization of ERBB3 with both EGFR and ERBB2, and activation of ERK and AKT downstream pathways, as well as cell proliferation and invasion.[231] Blockade of the EGFR with gefinitib prevented NRG stimulation of EGFR/ERBB3 hetero-dimers, ERK activation and cell proliferation, but ERBB2/ERBB3 heterodimers, AKT activation and cell invasiveness were persistently induced by NRG, illustrating the involvement of multiple pathways engaged by NRG-activated ERBB3.

Thus many results are consistent with NRG-stimulated ERBB3 having an important role in the malignant properties of mammary carcinoma cells. However, while high expression of NRG in mammary cancers may often contribute to their malignant phenotype,[224] a universal pro-tumorigenic role for NRG in mammary cancer cannot be assumed. During development of the mammary gland NRG1α is the main form expressed, and is necessary for differentiation as well as proliferation.[82] NRG caused differentiation in MDA-MD-453 and AU565 mammary cancer cells, and several NRG isoforms resulted in cell cycle arrest, differentiation or apoptosis, particularly in cells with high ERBB2 expression such as AU565, which lacks ERBB4.[232] Signaling pathways involved included p38 MAPK, PKCα, mTOR, JNK, caspases 7 and 9 and downregulation of BCL-2.[232]

In a series of cell lines of increasing malignancy derived from MFC10A cells, in the nontransformed cells, which do not express NRG or ERBB4, added NRG was anti-proliferative but acquisition of the fully malignant phenotype correlated with presence of high levels of secreted NRG.[184] Thus even within the same lineage the qualitative effect of NRG differed. Furthermore, there are numerous NRG isoforms, each of which may have unique functional implications.[81] The persistence of the NRG cytoplasmic tail in certain isoforms may relate to apoptotic effects.[82] To complicate matters further, invasiveness can be increased by NRG in SK-BR-3 breast cancer cells even while proliferation is suppressed.[233]

There is little information on NRG-independent activation of ERBB3 in mammary carcinoma cells. In SK-BR-3 mammary cells, which do not produce NRG, there is constitutive activation of ERBB3 and complex formation with ERBB2,[143,175] suggesting possible transactivation via the EGFR and/or ERBB4, facilitated by expression of large amounts of ERBB2. The environmental xenoestrogen β-hexachlorocyclohexane caused complex formation between ERBB3 and ERBB2 in MCF-7 mammary carcinoma cells and, interestingly, did not result in ERBB3 phosphorylation, though the chemical synergized with the ERBB3-activating effects of NRG.[234]

Summary and future for ERBB3 in breast cancer—These many results offer a complex picture of ERBB3 in breast cancer, with much data pointing to its active involvement, but also

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some ambiguities. The collective evidence is summarized in Table 5. ERBB3 may interact in several ways with breast cancer therapy. It contributes to tamoxifen resistance by unknown mechanisms.[235] There are compelling new results showing that ERBB3 upregulated activity is a means of escape from therapeutic suppression by several tyrosine kinase inhibitors, in at least six mammary cancer cell lines, by a novel pathway involving feedback from AKT.[127,236] ERBB3 is likely to become increasingly a center of attention for breast cancer treatment.

Ovarian cancer

Emerging evidence implicates ERBB3 in other cancers of endocrine-responsive tissues, including ovary and prostate. Interesting features are summarized in Table 6. In a comparative genomic hybridization study of ovarian serous adenocarcinomas, the *ERBB3* gene was found to be amplified 2.4- to 3-fold.[237] ERBB3, as well as NRG, is expressed in the majority of ovarian tumors, with highest frequency in carcinomas.[238–245] Although greatest expression was reported in early-stage or more differentiated tumors,[239–241] association of ERBB3 with poor prognosis has also been reported, for transitional cell carcinoma[240] and endometrioid cancers.[237] It has recently been confirmed that high ERBB3 expression in ovarian cancer correlates with poor survival.[246] In the latter immuno-histochemical study, ERBB3 overexpression was more common than high ERBB2 and the predominant localization of the ERBB3 protein was cytoplasmic, in contrast to the typical membrane staining for ERBB2.

In some ovarian cancer cell lines, responses of ERBB3 to NRG have seemed to be mediated by formation of heterodimers with ERBB2, as observed for mammary cancer cells (above).[208,243] However, there have also been some unique observations pertaining to ERBB3 in ovarian cancer cells. In the cell line OVCAR3, activation of ERBB3 and its association with PI3K p85 were independent of ERBB2.[128] This was confirmed by a later study with this cell line showing that ERBB3 and ERBB4, but not ERBB2 or EGFR, were phosphorylated after NRG treatment.[247] In this cell line, ERBB4 could have effected transactivation of ERBB3. However, in SKOV3 and IGROV1 cells, which lack ERBB4, NRG activated ERBB3 without any effects on ERBB2 or EGFR.[247] In OVCAR3 cells, EGF led to activation of all four ERBB family members, but in the cell line OAW42, lacking ERBB3, EGF did not activate ERBB4 and NRG activated ERBB4 only after long exposure. These results indicate, for ovarian cancer cells, a central role for ERBB3 in activation of ERBB4, and suggest that ERBB3 itself is activated by a path other than the other ERBB receptors, either its own very weak kinase activity, or by recruitment of some other kinase known to increase its activation, such as PTK6[125] or c-SRC.[126]

Another intriguing observation was the presence of truncated *ERBB3* transcripts of 1.6, 1.7, 2.1 and 2.3 kb in ovarian carcinoma cells lines.[23] When cloned into fibroblasts, three of these made truncated proteins, including several that were secreted and one that was retained intracellularly.

Prostate cancer

Development and progression of prostate cancer involves complex contributions from both the androgen receptor and AKT-regulated pathways, and interactions between these signaling components.[248–250] Furthermore, ERBB2/ERBB3 complexes caused AKT-independent phosphorylation of the androgen receptor that stabilized the protein and enhanced its transcriptional activity.[248] Increased expression of ERBB3 in prostate cancers, compared with normal prostate, has been demonstrated by immunohistochemistry in several studies[79,251–254] and was associated with poor prognosis.[252] Microarray analyses have likewise shown an increase in *ERBB3* mRNA in these cancers.[254–256] *ERBB3* was one of 15 genes whose expression levels had promise as diagnostic and prognostic markers for prostate cancer; *EGFR* and *ERBB2* were also in this cluster.[256] In a particularly sophisticated and thorough

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microarray-based study of nonmetastatic prostate cancers, increased *ERBB3* mRNA was confirmed in laser microdissected samples.[254] *ERBB4* mRNA was also overexpressed in these prostate cancers, whereas *EGFR* and *ERBB2* were less frequently affected, and it was concluded that ERBB3/ERBB4 may be particularly important. Other studies however have failed to confirm a close correlation between ERBB3 and ERBB4 in prostate cancers[253] and in prostate cancer cell lines ERBB4 is frequently not expressed.[257–259]

ERBB3 may be activated in prostate cancer cells by NRG, leading to formation of ERBB2/ ERBB3 hetero-dimers. ERBB2 has been strongly implicated in prostate cancer.[260–264] Findings regarding NRG expression have been mixed. While NRG1 was expressed in the majority of prostate cancers as observed by immunohistochemistry,[252] other studies have found this ligand to be absent from prostate cancers[253] and malignant cell lines,[253,258] although expression of NRG in prostate stroma[253] and other cell types could have effects on cancers within the organ.

Reported effects of NRG are likewise varied. In prostate cancer cell lines, added NRG led to ERBB3/ERBB2 activation and triggering of several downstream signaling cascades, including activation of PI3K, and in the androgen-responsive cell line LNCaP caused differentiation and reduced growth.[258] Growth suppressive effects of multiple isoforms of NRG were confirmed for LNCaP cells,[253] whereas growth of two androgen-nonresponsive lines, DU145 and PC3, was not affected by these ligands.

In contrast, in the androgen-independent prostate cancer cell line 22Rv1, NRG stimulated ERBB2/ERBB3 complex formation, ERBB2 phosphorylation and cell proliferation, effects that were reduced by application of the 2C4 monoclonal antibody which blocks complex formation by ERBB2.[265] Similar results were reported for 22Rv1 as well as other androgen-independent prostate cancer cells in another investigation.[212] In the CWR-R1 recurrent prostate cancer cell line, there was evidence for an autocrine pathway involving NRG and low-level constitutive ERBB2/ERBB3 activation leading to androgen receptor transactivation.[266] Xenografts of three androgen-dependent cell lines (CWR22, LNCaP and LNCaP35) also showed growth inhibition under treatment with 2C4 monoclonal antibody, although effects of NRG on these cells in culture were not reported.[212]

One of the consequences of NRG activation of ERBB3 is release of the EBP1 protein. Among the several known effects of the freed EBP1 is interaction with the androgen receptor as an inhibitor, so in androgen-dependent prostate cancer cells EBP1 suppressed proliferation and xenograft growth, in part by blocking androgen action.[162–164] Thus in such cells there is theoretical possibility of opposite effects of NRG activation of ERBB3: activation of the androgen receptor and its effects via AKT-dependent and -independent actions, and inhibition of it via EBP1. It is not surprising that variable effects have been seen experimentally with NRG applied to prostate cancer cells.

ERBB3 may also be activated via the EGFR in prostate cancer cells. Development of an autocrine or paracrine TGFα/EGFR growth-stimulatory pathway in prostate has been uniformly observed by many investigators (for example see Culig Z *et al*.[267]). Inhibitor studies indicated that TGFα and EGF contribute to cell proliferation in hormone responsive LNCaP cells;[259,268] treatment of these with EGFR ligands activated the ERBB3-PI3K p85-AKT pathway, and inhibition of PI3K led to apoptosis.[269] EGF stimulated growth of three of four prostate cancer cell lines; only the poorly differentiated line PC3 was refractory.[259] Interestingly, in primary prostate cancers acquisition of autocrine coexpression of both TGFα ligand and EGFR was a characteristic of androgen-independent metastases.[270] As noted above, high expressions of *EGFR* and *ERBB2*, as well as *ERBB3*, mRNAs in microarrays were indicators of prostate cancer,[256] and all were expressed and constitutively phosphorylated in

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hormone nonresponsive prostate cancer cell lines PC3 and DU145, whereas in LNCaP there was low constitutive phosphorylation of EGFR and ERBB3.[271] Expression of mRNAs for TGFα, as well as for *amphiregulin*, *heparin-binding EGF* and *epiregulin*, were 10- to 100-fold greater in androgen-independent DU145 and PC3 cells, compared with hormone-responsive LNCaP and PNT1A cells.[272] In addition, low levels of EBP1 in androgen-independent prostate cancer cells[164] could contribute to constitutive upregulation of androgen receptor-controlled events.

Whereas ERBB3 is clearly activated via the EGFR to send survival signals through PI3K in hormone-responsive LNCaP prostate cancer cells,[269] effects of ERBB2/ERBB3 on androgen receptor stability were independent of EGFR and did not involve AKT.[248] The upstream activator of ERBB2/ERBB3 and the responding downstream kinase(s) involved in this novel scenario remain to be demonstrated. Other crosstalk may also be important. For example, in LNCaP cells, interleukin-6 treatment activated ERBB2 and ERBB3 without involvement of the EGFR, in a process apparently involving the IL6 receptor.[273]

Finally, additional complexity is added by prostate cancer-related differences in the intracellular localization and in the processing and secretion of ERBB3. Prostate cancers, especially hormone refractory ones, show increased nuclear ERBB3, but in cancer cell lines nuclear ERBB3 was more notable in the hormone-sensitive ones,[79] and biochemical recurrence of prostate cancers was significantly associated with reduced nuclear localization.[274] A secreted isoform of ERBB3 was identified in 41/45 prostate cancer bone metastases and in activated osteoblasts and new bone matrices, but not in epithelial cells of primary cancers.[26] This secreted isoform stimulated bone cells to express osteonectin, which enhanced the invasiveness of the prostate cancer cells.[275] Furthermore, in xenograft experiments, a bone microenvironment, as compared to subcutaneous tumors, promoted nuclear localization of the ERBB3, as did castration of mice bearing subcutaneous tumors.[276]

Interesting findings regarding ERBB3 in prostate cancer are summarized in Table 6. The main conclusion that can be drawn from this tantalizing tangle of findings at present is that ERBB3 is very likely an important player in prostate cancer, and contributes in complex ways. In the practical context of potentially using ERBB3 as a molecular target for treatment of prostate cancer, it seems particularly important to sort out the growth-suppressive, differentiation-promotive vs the mitogenic, pro-survival effects of NRG and the involvement of ERBB3 in these.

Kidney and urinary bladder

Although an early study failed to detect ERBB3 in six renal cell carcinomas,[277] this protein was found in 28% of urothelial carcinomas; ERBB2, but not ERBB3, correlated with tumor invasiveness and survival.[278] In bladder cancers, ERBB3 was highly expressed in 20%, and had a possible positive correlation with EGFR and a negative one with ERBB2.[279] More recent studies have indicated that ERBB3 enhances survival in the context of bladder cancer, with some interesting and complex relationships among the ERBB family members and their ligands. Low mRNA expression for *ERBB3*, *NRG2*α, *NRG2*β and *NRG4* correlated with invasiveness, and high *ERBB3* and *NRG4* expressions were associated with favorable prognosis, especially where *ERBB3* or *ERBB4* were highly expressed along with *NRG4*.[280] This was confirmed in a further study, where high expression of *EGFR* or *ERBB2* predicted poor survival only when *ERBB3* and *ERBB4* had low expression.[281] RT4 bladder carcinoma cells were treated with mitogenic HB-EGF, resulting in increased mRNA expression of *ERBB3*, *ERBB4*, *NRG1*α and *NRG1*β, whereas expressions of *NRG2*α, *NRG2*β and *NRG4* were decreased.[282] These findings point to positive and negative regulatory interactions involving all four ERBB family members and several NRGs in cancers of this cell type.

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Hematopoietic neoplasms

There have been few studies of ERBB3 in this class of neoplasms. No measurable ERBB3 was found in seven unspecified hematopoietic cell lines.[17] In multiple myeloma cells, interferon α treatment led to phosphorylation of ERBB3, and silencing ERBB3 with siRNA reduced the growth response both to the interferon and to interleukin-6.[129] Several Janus kinase family members, TYK2 and JAK1, were found to be involved in the transactivation of ERBB3 by the interferon receptor 1.[131]

Nervous system

ERBB3 in normal nervous system—In fetal rats Erbb3 expression was strong in ventral roots of the spinal cord.[283] In mouse cerebellum Erbb3 was detected by western blot only after birth, peaking at postnatal day 18; *in situ* hybridization showed it to be localized to granule cells, probably associated with the process of maturation of synaptic connections.[284] Although *Erbb3* mRNA was not detected in fetal mouse brain,[34] studies with *Erbb3* knockout mice revealed an essential role in neurological development; these mice exhibited severely underdeveloped sympathetic ganglia and partial lack of Schwann cells. Erbb2/Erbb3 heterodimer was shown to be necessary for Schwann cell differentiation.[286–288] Failure of migration of progenitor cells from neural crest was similarly observed in *Erbb3*, *Erbb2* and *Nrg* knockout mice, implying a role for Nrg to Erbb3/Erbb2 signaling.[285,289,290]

ERBB3 also has an essential role in development of the human nervous system: lethal congenital contractural syndrome 2, an autosomal recessive trait associated with atrophy of the anterior horn of the spinal cord, is caused by aberrant splicing of *ERBB3*.[291]

In adult rodent brain *Erbb3* mRNA, with prominence mainly in white matter, has an expression pattern different from that of *Erbb4*.[34,292,293] While *Erbb3* mRNA was expressed in the ventral and dorsal spinal cord roots of fetal rats, it was absent from these areas in adults. It re-appeared after ventral funiculus lesion; a role for Erbb3 in regenerative growth of axons was suggested.[283] Erbb3/Erbb2 appear to contribute to peripheral nerve regeneration in rats.[294] Erbb3 may also retain a role in mature Schwann cells during reparative proliferation, as indicated by evidence for Nrg/Erbb3 autocrine loop for Schwann cell mitogenesis in culture,[295] and association of upregulation of Erbb2/Erbb3 in Schwann cells post axotomy.[296]

ERBB3 and brain cancer—ERBB3 and the transcription factor SOX10, which regulates ERBB3 directly or indirectly in neural tissue, are notably overexpressed in pilocytic astrocytoma (a common childhood glioma), compared with other pediatric brain tumors.[297,298] Since SOX10 is an embryonic neural regulator of ERBB3, these childhood neoplasms may reflect a dysregulated developmental pathway. SOX10 was expressed in three-fourths of schwannomas, and in relatively differentiated neoplasms, for example in schwann-like cells of neuroblastoma (all ganglioneuromas and some stage IV neuroblastomas), correlated with widespread expression of ERBB3.[299] Interestingly, SOX10 and ERBB3 were rarely expressed in pediatric glioblastomas, but were consistently seen in radiation-induced glioblastomas, and gene expression patterns of the latter cancers resembled those in pilocytic astrocytomas.[300]

ERBB3 was rarely expressed in meningiomas.[301] Most studies have reported expression, though not amplification, in adult gliomas,[301–304] with one exception.[305] In astrocytic glioma cell lines, constitutive ERBB3 phosphorylation, complex formation with ERBB2 and activation by NRG occurred to varying degrees and was associated with inhibition of apoptosis rather than stimulation of mitosis.[302,306]

ERBB3 in retinoblastomas—In a micoarray analysis of 10 childhood retinoblastomas compared with adult normal retina, *ERBB3* expression was increased 9.9-fold; this was

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confirmed by RT-PCR.[307] *PI3K*, *class 3* and *AKT1* were also increased. Interpretation of this study is complicated by lack of availability of normal infant retinas as reference controls.

Melanomas

Melanomas, as derivatives of neural crest, have ontogenetic kinship to glial cells. In several microarray clustering studies, *ERBB3* has emerged as one of a small number of genes whose upregulation is characteristic of melanoma and tumors with melanocytic features.[308–311] Immuno-histochemical analysis of ERBB3 protein confirmed these results in one series of studies and suggested increased ERBB3 expression associated with metastatic progression.[312,313] Although another investigation reported that ERBB3 was found in only a minority of melanomas, and only in those that were not metastatic,[314] and several melanoma cell lines did not express ERBB3,[315] ERBB3 showed a fourfold increase in expression in melanoma micrometastases and 14-fold increase in macrometastases, compared with normal lymph nodes.[316] NRG-stimulated migration but not proliferation of melanocytes, and had the opposite effect in two melanoma cell lines,[317] suggesting qualitative changes in ERBB pathway signaling during melanoma development. Muc4, a transmembrane mucin that promotes growth and metastasis of melanoma cells, caused increased membrane localization and stability of ERBB2 and ERBB3.[318]

Clear cell sarcoma of soft tissue is a rare tumor of children and young adults with melanocytic differentiation. Upregulation of ERBB3 is particularly prominent in these cancers.[309,310] Cell lines derived from these tumors expressed ERBB3 protein and either ERBB2 or ERBB4; in half of the lines ERBB3 was constitutively activated by NRG1 expression; the others were responsive to added NRG1.[319]

Gastrointestine (Table 7)

Expression and function in normal gastrointestinal tissues—ERBB3 protein was detected by immunohisto-chemistry in epithelial cells throughout the gastrointestinal tract, including squamous epithelium of the oropharynx and esophagus, parietal cells of the stomach and surface enterocytes of small and large bowel.[320,321] NRG1 on the other hand was detected in mesenchymal but not epithelial cells of gastric mucosa.[321] In mouse fetuses Erbb3 was expressed in the gastric epithelium, which was much thinned in the *Erbb3*$^{-/-}$ knockout mice;[289] in fetal pancreas Erbb3 was expressed in the mesenchyme, not the epithelium and in the knockout fetuses pancreatic development was retarded. In rat hepatocytes Nrgβ1 bound specifically to Erbb3, induced its phosphorylation, and increased DNA synthesis[322] and insulin inhibited Erbb3 expression.[322] This was also observed *in vivo* under conditions of insulin insufficiency, and the PI3K pathway was implicated in the insulin effect.[323]

Colorectal cancers—*ERBB3* is occasionally mutated in colon carcinomas, with two mutants found in 100 cases analyzed.[324] Increased *ERBB3* mRNA or protein is more common, detected in 34–90%.[320,325–330] Association with poor clinical outcome was noted in one study[328] but not in several others.[329–331] In a recent and particularly complete investigation,[332] colonic adenomas expressed more cytoplasmic ERBB3 than did normal tissues or carcinomas, whereas nuclear staining was observed in 82% of normal, 54% of adenomas and 23% of carcinomas, all significant differences. Nuclear ERBB3 or pERBB3 did not, however, correlate with any tumor characteristics. However, *ERBB3* mRNA levels were higher in cases with positive lymph nodes, and correlated significantly with reduced time-to-disease progression and probability of relapse.

Coexpression of ERBB3 with EGFR and ERBB2 was frequently noted in these investigations, and there is evidence that both of these receptors contribute to ERBB3 activation in colon cancers. In colorectal cancer cell lines, ERBB2 and ERBB3 generally show high expression

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and constitutive activation and dimer formation,[141] which is further enhanced by treatment with NRG, leading to stimulation and enhanced invasiveness.[333] Stimulation of COX-2 gene expression was part of the mechanisms of these effects of NRG. The EGFR may also be important, as sensitivity of colon cancer cell lines to growth suppression by the EGFR-specific inhibitor erlotinib (Tarceva) correlated closely with expression of ERBB3.[334] As further evidence for ERBB3's importance in this type of cancer, inhibition of proliferation of HT-29 colon cancer cells by conjugated linoleic acid involved downregulation of the ERBB2/ERBB3, PI3K, AKT pathway.[335]

Pancreatic cancers—In pancreatic cancers, *ERBB3* mRNA or protein has consistently been observed to be increased[336–339] and associated with advanced stage and poor outcome.[337,339] As with colorectal carcinoma cell lines, pancreatic cell lines' sensitivity to inhibition by the EGFR-specific inhibitor erlotinib was determined by coexpression of ERBB2 and ERBB3, and in particular the level of constitutive pERBB3.[334] Activation of AKT and S6, but not of ERK, was specifically linked to this ERBB3 effect, and results were confirmed by down-regulation of ERBB3 by siRNA. Similarly specific downregulation of ERBB2 by an HSP90 inhibitor resulted in radiosensitization only in pancreatic cell lines that did not express ERBB3, and downregulation of ERBB3 by siRNA in other pancreatic cell lines resulted in acquisition of radiosensitivity, related to reduced tyrosine phosphorylation of EGFR.[340]

Gastric cancers—ERBB3 showed increased expression in gastric cancers.[277,341,342] Both membrane and cytoplasmic staining were noted, whereas elevated ERBB3 in surrounding tissue was mainly cytoplasmic.[341] Relative expressions of EGFR and ERBB2, as well as ERBB3, were higher in gastritis compared with normal stomach and higher yet in carcinomas; only carcinomas had high expression of all three receptors.[342] Gastric cancer cell lines all expressed ERBB3 and a truncated, secreted product.[22] Such lines expressed EGFR and ERBB2 as well, but not ERBB4 or NRG1.[321] Addition of NRG1 led to cell proliferation and formation of pERBB3, ERBB3/ERBB2, ERBB3/EGFR and ERBB2/EGFR dimer formation, and p85 PI3K association with ERBB3. ERBB3 activation was also seen during coculture with gastric fibroblasts that secreted NRG1. These results suggest that activation of an ERBB3-dependent mitogenic pathway in gastric cancer may involve NRG1 paracrine stimulation from mesenchymal cells.

Several NRG isoforms had a marked morphogenic effect on gastric carcinoma cells, with motility as the fundamental cellular response; ERBB3/ERBB2 complexes appeared to mediate this response.[343] Strong evidence for a key role for ERBB3 in gastric malignancy came from a study of poorly differentiated signet-ring cell gastric carcinomas.[344] Activation of ERBB3 and complexation with PI3K were associated with the de-differentiated state; expression of a chimera of activated ERBB2/ERBB3 resulted in increased malignancy of an initially highly differentiated cell line.

Head and neck cancers—In squamous cell and verrucous oral carcinomas ERBB3 has been reported to be highly expressed and associated with invasiveness, metastasis and poor prognosis,[345–351] although other studies of oral cancer have not found ERBB3 to be overexpressed[352] or related to survival.[353] Coexpression of ERBB3 with EGFR and ERBB2[348] or of ERBB2 and ERBB3[349] related to poor prognosis, and expression of ERBB3 was correlated with resistance to the EGFR inhibitor gefitinib.[354] Increases in ERBB3 along with EGFR and ERBB2 were also reported for papillary carcinoma of thyroid.[355,356]

Involvement of ERBB3 in oral cancer was confirmed in a rat model, where Erbb3 protein was increased in carcinogen-induced oral carcinomas.[357] In diabetic rats, this protein was increased in hyperplastic and dysplastic lesions also.

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A role for ERBB3 may be less likely for esophageal cancers. EGFR is often overexpressed in these cancers.[358–360] *ERBB3* mRNA on the other hand was significantly lower than normal, even though immuno-reactivity was high in 64% of tumors; there was no relationship with tumor characteristics or prognosis.[358] The highly expressed EGFR in esophageal cancers was sensitive to inhibitory effects of gefitinib, and both the ERK and the PI3K pathways were involved,[360] but activation of the AKT isoforms after treatment of esophageal carcinoma cell lines with EGF did not entail activation of ERBB2 or ERBB3.[359]

Respiratory tissues

Role of ERBB3 in normal respiratory tissues—Type II alveolar lung cells from fetal rats expressed more Erbb3 than did fibroblasts.[361] The Erbb3 coimmunoprecipitated with both Erbb2 and Erbb4, and had a prominent nuclear localization, moving into the cytoplasm after NRG stimulation. ERBB3 (and colocalized ERBB2 but not ERBB4) were demonstrated in airway epithelium from explanted human fetal lung.[362] NRG treatment caused a decrease in production of SP-A, a differentiation marker, in type II alveolar cells of these lungs. Stimulation of type II cell proliferation was observed in this same study. Thus at least in human fetal lung epithelium, NRG to ERBB3/ERBB2 signaling favored cell division while suppressing differentiation programs. A similar conclusion was reached with the BR516 cell line from neonatal rat distal airway, which was characterized by high mRNA for *Nrg, Egfr, Erbb2* and *Erbb3*, but little for *Egf, betacellulin*, or *Erbb4*.[363] These cells responded to Egf, betacellulin or Nrg with growth and a probable autocrine effect of Nrg occurred.

This same signaling loop may regulate regenerative proliferation of adult lung epithelium. ERBB3 was expressed in primary proliferating cultures of human bronchial epithelium.[364] In scrape-wounded adult lung epithelial monolayers, tyrosine phosphorylation of EGFR, ERBB2 and ERBB3 occurred immediately.[365] Smoking was associated with significant increases in EGFR and especially in ERBB3 in bronchial epithelial cells compared to nonsmokers.[366] In intact polarized human airway epithelium, NRG1 and ERBB3 were segregated, the former on the apical membrane, and the latter on the basolateral surface.[367] When the epithelium was disrupted, as in lung injury, NRG1 contacts and activates ERBB3 (and ERBB2). Cell division could then ensue to re-establish epithelial integrity.

There is additional complexity, however, related to interactions between epithelial cells and fibroblasts in lung. Paracrine production of NRG by fibroblasts led to surfactant production by type II cells.[368] Similarly differentiation was induced in primary human lung epithelial cells when NRG was added to the basolateral medium.[369] Coculturing of airway epithelial cells with primary lung fibroblasts, which expressed all ERBB ligands except betacellulin, also resulted in epithelial differentiation. Blocking ERBB2 activation with trastuzumab caused de-differentiation of well-differentiated human airway epithelial cells. It was proposed that ERBB2 stimulation is essential for maintaining epithelial differentiation and hypothesized that ligands secreted by mesenchyme underlying the airway epithelium may be involved in maintaining epithelial differentiation. Thus, the effects of ERBB3 activation in peripheral lung may differ, perhaps depending on whether the ligand comes from an autocrine or a paracrine source and could well reflect the integrated activities of several ligands and pathways.

Further, ERBB3 signaling in lung epithelial cells may affect surrounding mesenchyme. In intact mice, lung injury by bleomycin resulted in Erbb3 activation as part of the response, along with inflammatory cell infiltration and collagen deposition.[370] In mice where Erbb3-mediated effects were blocked in lung by a dominant-negative *Erbb3* transgene, these outcomes were diminished after bleomycin and survival was improved. Blockade of Erbb2/Erbb3 signaling with the 2C4 monoclonal antibody had a similar effect.[371] These results imply that, at least in this model, Erbb3-initiated signaling in epithelial cells ultimately results in increased collagen

production by mesenchymal cells, leading to fibrosis. Erbb3 was also implicated in chronic obstructive pulmonary disease in rats.[372]

In normal human nasal epithelium ERBB3 expression was demonstrated by immunohistochemistry and by RNA analysis and immunoblotting of nasal epithelial cells in primary culture.[373] EGFR and ERBB2 but not ERBB4 were also noted, as well as EGF, TGFα, heparin-binding EGF, amphiregulin and betacellulin.

ERBB3 in lung cancer (Table 8)

ERBB3 expression and lung cancer—An immunohisto-chemical study identified ERBB3 protein in alveolar type II and bronchioalveolar cells of normal lung regions in operated lung cancer patients, where staining was less intense and more diffuse in the cytoplasm, compared with punctate cytoplasmic and/or membrane staining in carcinoma cells.[374] As measured by immunohisto-chemistry, ERBB3 was highly expressed in some lung adenocarcinomas[277] and associated with poor prognosis.[274] Quantitative real time RT-PCR indicated that high *ERBB3* expression was significantly associated with decreased survival in patients with early stage (I–IIIA) NSCLC.[375] Coexpression of ERBB3 with other ERBB family members was indicative of tumor recurrence.[376] The expression of the proliferation-associated marker Ki-67 at a higher frequency in ERBB3-positive NSCLC cases than in ERBB3-negative tumors was suggestive of a contribution of ERBB3 to aggressive behavior; combination of elevated ERBB3, p53 and microvessel density predicted poor survival.[377] In an interesting real-time PCR-based study of expression of 56 receptor tyrosine kinases in early stage NSCLC, *ERBB3* was one of 10 associated with metastasis development and decreased survival, along with *EGFR* and *ERBB2*.[375] Microarray analysis of NSCLC yielded a five-gene signature that predicted relapse-free and overall survival, and *ERBB3* was one of these five genes.[378] There is therefore mounting evidence that ERBB3 expression supports lung malignancy.

Additional strong evidence for the importance of ERBB3 expression in lung tumorigenesis came from ERBB3 transgenic mice, which developed a high incidence of lung adenocarcinoma compared to nontransgenic mice,[379] in spite of lack of K-*ras* mutations. Mice doubly transgenic for *ERBB3* and *Erbb2* had an incidence of spontaneous lung tumors similar to that in *ERBB3* singly transgenic mice, but developed larger tumors with a shorter latency, suggesting that Erbb2 synergized with ERBB3 in lung tumor progression. More tumors with shorter latency also occurred in *ERBB3* transgenic mice treated with the carcinogen methylnitrosourea, which induced K-*ras* mutations, indicating a possible promotion/progression effect of high ERBB3 expression on tumors initiated by genotoxic damage such as K-*ras* mutation.

Another important mouse model study utilized transgenic expression of mutant K-ras for lung tumor initiation.[380] Erbb3 was not present in normal lung cells, but increased steadily during the different stages of lung tumor progression. Amphiregulin, epigen and epiregulin showed increased mRNA expression in the lungs of the K-ras^{LA1} mice, whereas EGF, TGFα, β-cellulin, NRG1 and NRG2 were not changed relative to wildtype. The EGFR inhibitor gefitinib treatment reduced the levels of Erbb3, amphiregulin and epiregulin in lung tumors and suppressed the growth of alveolar neoplasia in these mice.[380]

ERBB3 mutation and amplification in lung cancer—No mutations in *ERBB3* have been detected in lung cancers.[324,381] Several studies have reported lack of *ERBB3* gene amplification in primary lung cancers and ERBB3-expressing lung cancer cell lines.[382–384] However, a recent fluorescent *in situ* hybridization analysis revealed *ERBB3* gene amplification (high polysomy and gene amplification) in 26.8% of the cases in a cohort of 82 NSCLC patients treated with gefitinib.[385] *ERBB3* genomic gain was significantly associated with female gender and nonsmoking status and not with tumor stage or histology. It was

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possible that this amplification could have been related to the chemotherapy that most of these selected patients had received.

ERBB3 expression and functions in lung cells in culture—ERBB3 was expressed in human bronchial cells in culture and in various types of lung cancer cells,[386] although its levels were much greater in a transformed compared with a nontransformed sister line of human bronchial epithelial cells.[387] By contrast, ERBB3 protein was absent from an immortalized, nontransformed cell line from human peripheral lung epithelium, but highly expressed in most human lung adenocarcinoma cell lines.[139] The same contrast was noted for nontransformed vs malignant mouse lung cell lines,[140] and is consistent with absence of Erbb3 from lung alveolar cells in intact mice.[380] These observations are further indicators that ERBB3 upregulation is distinctly related to neoplasia development in the peripheral lung.

The pathway involving ERBB3 has been explored in detail in mouse and human lung adenocarcinoma cell lines,[139,140] providing the first evidence that abnormal expression of ERBB3 has a controlling role in growth and invasiveness of these cells and defining an intracellular signaling pathway leading to these effects. Like ERBB3, TGFα was highly expressed in the mouse and human lung adenocarcinoma cell lines, but not in their nontransformed counterparts.[139,140] TGFα expression has also been strongly implicated in transformation of bronchial epithelial cells.[386,387] Use of specific inhibitors established the signaling sequence: TGFα, EGFR, ERBB2/ERBB3, p85 regulatory subunit of PI3K, AKT, GSK3β inactivation, cyclin D1 increase and cell cycle progression. The role of ERBB3 was proposed to be complex formation with and activation of p85, and this was confirmed by use of an *ERBB3*-specific antisense oligonucleotide, which reduced amounts of ERBB3-p85 complex and significantly suppressed cell proliferation only in ERBB3-expressing human lung cancer cells.

NRG1β also activated this pathway in the lung adenocarcinoma cells. Expression of NRG1α and β at the RNA level was described in four human lung cancer lines; only NRG1α protein was detected in lysates and in conditioned medium.[388] An ERBB3-specific antibody, which blocks the NRG-binding sites on ERBB3, reduced NRG-induced ERBB2/ERBB3 activation significantly and transfection of DN ERBB3 abrogated NRG-induced ERBB2 phosphorylation in a dose-dependent manner.

The findings cited above implicate AKT as a critical mediator of ERBB3 effects in lung cancer cells, activated via PI3K. Accumulating evidence supports this role for the PI3K/AKT pathway.[389–392] The PI3K–AKT pathway was important in actions of tobacco carcinogens and in transformation-related characteristics of lung cells.[393,394]

The JAK-STAT pathway may also be involved in NRG stimulation of proliferation of lung cells mediated by ERBB3. In two human lung cancer cell lines, NRG1 had a modestly stimulatory effect on cell number, and involvements of ERBB2, JAK3, TYK2, STAT3 and STAT5 were indicated.[153] However, direct association of the JAK family members with ERBB proteins was not demonstrated.

Small interfering RNAs are proving to be highly useful tools for experimental downregulation of specific genes and are exciting interest for therapy. In one of the first applications of siRNA as a potential therapeutic approach for lung cancer, we found that siRNA for ERBB3 applied to human lung adenocarcinoma cells could stably and dose-dependently reduce cell numbers in A549, H441 and H1373 cell lines and inhibit soft agar growth, motility, migration and invasiveness.[140] Apoptosis, necrosis and suppression of cell cycle contributed to the reduction in cell number. siRNAs to AKTs were also tried in this study. There are three AKT isoforms; the functional significance of these in lung is not known. In our investigation, *AKT1*, *AKT2*

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and *AKT3* siRNAs, like *ERBB3* siRNA, had suppressive effects on growth, necrosis, apoptosis and soft agar growth. siRNAs against all three isoforms had similar effect on apoptosis, necrosis and cell survival. *AKT2* siRNA was particularly effective in blocking migration and invasiveness whereas *AKT1* siRNA had no effect on cell migration or invasion. Thus AKT2 may be a particularly attractive potential target for therapy.

siRNAs to *ERBB3* or *AKT2* have recently been tested *in vivo* for effects on human lung adenocarcinoma xenografts, a model which has been shown to be predictive of clinical activity.[395] In three separate experiments, both siRNAs reduced by 40–80% the size of tumors formed by human lung adenocarcinoma A549 cells as xenografts in nude mice (Table 9). Nonsilencing siRNA was without significant effect. These findings are particularly remarkable, as the intravenous siRNA was administered as a saline solution without carrier.

ERBB3, EGFR mutation and clinical responsiveness to EGFR inhibitors— Recently activating mutations have been found in the *EGFR* gene in lung cancer patients responsive to EGFR tyrosine kinase inhibitors, including gefitinib and erlotinib.[396–399] These mutations are especially prevalent in non-smokers, females, East Asians and bronchioalveolar carcinomas.[400–405] EGFR polysomy/amplification and high mRNA and protein expression were also associated with survival after treatment with these inhibitors.[406–409] In PC-13 cells, which have no endogenous EGFR expression, transfected mutant EGFR showed high constitutive phosphorylation of itself and of AKT and STAT3, and prolonged cell survival under serum-free conditions.[410] Most recently, erlotinib[411] and gefitinib[412] are showing efficacy as first-line therapy for non-small cell lung cancers.

Emerging evidence indicates that not only EGFR activity, but also participation of other ERBB family members, especially ERBB2 and ERBB3,[409,413,414] are critical components of lung cancer clinical responsiveness to tyrosine kinase inhibitors.[409,415] As detected by immunohistochemistry, ERBB3 expression levels were higher in tumors of patients, who had shown an objective gefitinib response or stabilization of disease compared to those with progressive disease; ERBB3 was in fact a better predictor of response than *EGFR* mutation.[380] Among Japanese patients with lung cancer, tumor *ERBB3* mRNA was significantly higher in those with *EGFR* mutations, as well as in cancers from women and from non-smokers.[77]

Studies with lung cancer cell lines have confirmed an integral contribution of ERBB3 in sensitivity to EGFR protein tyrosine kinase inhibitors. Some lung cancer cell lines with wildtype EGFR show responsiveness to gefitinib or erlotinib. ERBB3 is high in those that are responsive,[392,416] in association with an epithelial as opposed to a de-differentiated mesenchymal phenotype.[417] Similarly, ERBB3, as well as epiregulin and amphiregulin, were expressed at higher levels in lung cancer cell lines that are highly (HCC827, H3255 and H4006) or moderately (H1819 and HCC2279) sensitive to gefitinib compared to the gefitinib resistant cell line H1299.[380] Association of responsiveness with levels of activated AKT[418–421] also suggested a central role for ERBB3. In gefitinib-sensitive lines with high expression of ERBB3, gefitinib led to uncoupling of ERBB3 from the p85 regulatory subunit of PI3K and downregulation of ERBB3 with shRNA markedly reduced AKT activation.[420] Engelman and Cantley[414] describe unpublished data that constitutive oncogenic mutants of PI3K/AKT abrogate response to gefitinib, confirming the pivotal role for ERBB3. Interestingly, gefitinib not only inhibits EGFR activity, but may also lead to sequestration of ERBB2 and ERBB3 as inactive heterodimers with EGFR.[422] Futhermore, coexpression of ERBB2 and ERBB3, in LK2 NSCLC cells with very low EGFR, may confer some sensitivity to gefitinib; evidently the affinity of the chemical for ERBB2 was increased 10-fold by its heterodimerization with ERBB3.[423]

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In spite of all of this evidence for participation of ERBB3 in gefitinib response, no change in the latter was observed in lung cancers showing *ERBB3* amplification and while *ERBB3* gene copies were significantly associated with gene gains for *EGFR* and *ERBB2*, they did not correlate with *EGFR* mutation or level of activated AKT.[385] ERBB3 protein was not analyzed in this study; it is possible that *ERBB3* copy number is not determining with regard to protein expression.

EGFR activity and ERBB3 expression are not the sole determinants of ERBB3's role in lung cancer. Forced expression of ERBB3 in H1299 and A549 cells did not increase sensitivity to gefitinib, and exogenous stable expression of WT EGFR or two EGFR mutants into H1299 cells did not render them sensitive to gefitinib.[380,420] A549 cells present wildtype, moderately amplified EGFR and moderate sensitivity to gefitinib. PX866, an inhibitor of the p110α catalytic subunit of PI3K, potentiated the antitumor activity of gefitinib against large A549 xenografts, giving complete tumor growth control in the early stages of treatment.[424] Additive effects of another PI3K inhibitor, LY294002, with gefitinib were also noted in H460 lung cancer cells.[421] These results suggest pathways to PI3K activation in addition to that controlled by EGFR. In A549 cells, as noted above, NRG can activate ERBB3, and ERBB3 siRNA blocked NRG-induced pAKT levels and increased cyclin D1 in A549 cells. These results together suggest that, at least in A549 cells, ERBB3 conducts proliferation and survival signals from both EGFR via ERBB2, and directly after NRG stimulation, again probably involving ERBB2 hetero-dimer formation.

Participation of an NRG/ERBB3 pathway in resistance to gefitinib has recently been confirmed by demonstration that gefitinib insensitivity in 44 NSCLC cells lines correlated very strongly with NRG expression and, much more weakly, with ERBB3.[425,426] Further, ERBB3 activation in these cells was correlated with levels of ADAM17, a sheddase for NRG, and siRNA inhibition of ADAM17 suppressed ERBB3 and AKT activation. Pertuzumab, a humanized anti-ERBB2 monoclonal antibody, is effective against ERBB2-expressing mammary and prostate cancer cells, but has varying activity in the context of lung.[427] In a panel of NSCLC cell lines, pertuzumab was effective in those wherein NRGα stimulated ERBB2/ERBB3 heterodimer formation and ERBB3 phosphorylation.

Yet another route for ERBB3 activation independent of the EGFR has recently been discovered: resistance to gefitinib acquired by NCSLC with mutant *EGFR* was due to amplification of the MET receptor, which in turn led to activation of ERBB3 and the PI3K and AKT down-stream-signaling pathways.[121,122] It may be that other pathways could also be involved: erlotinib-sensitive lung carcinoma cells expressed higher levels of the SRC-like kinase BRK; BRK is known to phosphorylate ERBB3 and promote PI3 kinase AKT signaling in mammary cells.[125] In addition, ERBB3 can be targeted independently of EGFR and ERBB2, as recently shown for the marine-derived anti-tumor agent kahalalide F.[428]

In short, ERBB3 not only participates in the sensitivity of the majority of lung cancers that respond to EGFR tyrosine kinase inhibitors, but also in other malignancy-associated signaling paths. These multiple facets increase its attractiveness as a molecular target for therapy in this type of cancer.

Conclusions and perspectives

The ERBB family of receptors, their ligands, and their many potential downstream signaling targets constitute a highly complex, layered network, requiring a systems biology approach.[6] Since it is clear that misbehavior of this network contributes to many cancers, it is essential to find the vulnerable nodes in this network for therapeutic applications. ERBB3 is rapidly emerging from its earlier disrespected categorization as a kinase-dead structural partner for

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ERBB2. It has become apparent that ERBB3 has a central, active role, indeed probably several discreet functions, in integrated cellular regulation. As detailed above, expression and activity levels of ERBB3 may determine the therapeutic effectiveness of tyrosine kinase inhibitory drugs for mammary, colorectal and lung cancers. Yet another mode by which ERBB3 influences the success of cancer treatment relates to targeting of the chaperone HSP90, which is a specific stabilizing agent for ERBB2. Ansamycin inhibitors of HSP90 were effective only in those breast cancers sustained by the ERBB2-ERBB3 pathway.[429] Similarly radiosensitization of cancer cells may be accomplished by chemical targeting of the chaperone HSP90, leading to downregulation of ERBB2 and loss of the EGFR signaling that engenders protection against radiation. However, if ERBB3 is highly expressed, EGFR/ERBB3 heterodimers allow persistence of this protective signaling. This apparent effect of high ERBB3 expression was demonstrated in pancreatic, prostate and mammary cancer cell lines.[340,430]

Thus, ERBB3 is an attractive therapeutic target in its own right and indeed may be an essential one as part of any treatment protocol focused on control of ERBB receptors or the PI3K/AKT pathway. ERBB3 is not an easily druggable target due to lack of kinase activity.[16] Several approaches for therapeutic targeting of ERBB3 have been tried experimentally (Table 10). RNA aptamers to the extracellular domain of ERBB3 inhibited NRG-induced ERBB3/ERBB2 heterodimerization, ERBB2 phosphorylation and growth of MCF7 breast cancer cells.[431] A synthetic designer zinc finger transcription factor inhibitory to *ERBB3* gene expression in A431 squamous cell carcinoma cells resulted in reduced proliferation and migration, and the repression of ERBB3 expression had a bigger effect than changing ERBB2.[432] The vitamin E isomer γ-tocotrienol inhibited mammary cell proliferation by specific block of ERBB3 activation and of downstream stimulation of the PI3K/AKT path-way. EGFR and ERBB2 were not affected; the mechanism of the specific action on ERBB3 is not known.[433] Micro-RNA 125a reduced ERBB3 RNA and protein, activation of AKT and cell growth and invasiveness of SKBR3 mammary carcinoma cells.[434]

Various other therapeutic approaches have been suggested. These include use of negative regulators of ERBB3 such as the NRDP1 ubiquitin ligase;[435] blocking of transactivation of ERBB3 or of nucleocytoplasmic trafficking of NRG;[436] and application of a specific inhibitor of ADAM17 sheddase.[426,437]

Our recent results with ERBB3 siRNA (Table 9) suggest that this may be a particularly simple and efficacious approach, as highly significant suppression of xenografted lung tumor growth was achieved with simple intravenous injection of saline solutions of siRNA. The potential importance of ERBB3 siRNA in therapy is underscored by recent results showing that downregulation of ERBB3 by siRNA in breast cancer cells abrogated their secondary resistance to tyrosine kinase inhibitors and allowed induction of apoptosis.[127] In view of the multiple ERBB3 ligands and the possibility of by-passing a block imposed by inhibiting or downregulating a single receptor,[438] simultaneous targeting of several ERBBs, for example by herceptin for ERBB2 and siRNA for ERBB3, could be explored.

To maximize the potential usefulness of ERBB3 as a therapeutic target, there are several intriguing aspects of the biology of ERBB3 that must be explored in more depth. Roles for the intracellular and secreted truncated forms of ERBB3 may be worth further study, especially in light of the recent demonstration that the p45 form is a prostate cancer metastasis factor.[26] The mysterious nuclear and nucleolar activities of ERBB3[76,78,79] must be unraveled. Further study is needed of the involvement of non-NRG, non-ERBB regulators of ERBB3, such as CDK5,[122–124] BRK,[125] SRC,[126] and MET.[120] The fascinating and widely expressed ERBB3 effector EBP1 has thus far been examined mainly in mammary and prostate cells;[154–164] may it be important also in other cancers where ERBB3 is clearly a player, such as lung and

melanoma? It is hoped that full understanding of the regulation and functions of ERBB3 will facilitate its integration into cancer management.

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Table 1

ERBB3 gene structure, mRNA, and protein characteristics and control

	References
Gene	
Human chromosome 12q13.2	19
23.2 kb, 28 exons	17,18
43–67% homology with other ERBBs	17,20,21
mRNA	
6.2 kb, with several alternative transcripts and truncated protein products	1,14,22–24
Positive regulation by AP transcription factor	35–37
Negative regulation by estrogen	38,39
Protein	
Extracellular ligand-binding domain consisting of four subdomains that change conformation in response to ligand	41,43,44,48
Ten potential glycosylation sites, at least one of which is critical to regulating heterodimerization with ERBB2	41,42
Absence of homodimer formation, but assembly of self-oligomers which are disrupted by NRG	52,53,55,57
High affinity for NRG, increased by heterodimerization with ERBB2	50
Cytoplasmic domain lacking kinase activity; unique amino acids in this domain affecting protein interactions	58,60
Thirteen tyrosines and a nuclear localization signal in carboxy terminal	1,17,18,20
Downregulation by slow endocytosis, followed by rapid recycling	63–65
Persistant ligand binding after endocytosis, at acid pH	50
Degradation and intracellular trafficking regulated by the ubiquitin ligase NRDP1, which affects cancer cell growth	67,68,70
Nuclear localization, dependent in part on NRG	76–80

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Table 2

Activation of ERBB3

	Reference
Ligands	
NRG1β most effective	84,86–88
Cell membrane clustering after NRG activation	95
Other receptors, heterodimer formation	
EGFR	101–108
ERBB2	106,110–113
ERBB4	78–92
MET	121,122
Other kinases	
Cyclin-dependent kinase 5 (CDK5)	122–124
c-SRC	118,119,126
BRK	125
Other (direct or indirect)	
AKT feed-back	127
Cell stress, TNFα, INFα	129,130
TYK2, JAK1	131

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Table 3

Protein SH2 or PTB domains interacting with pTyr sites in ERBB3 with high affinity (dissociation constants <1000 nM*, <500 nM**, <100 nM***)

Domain source	pY868	pY1054	pY1197	pY1222	pY1260	pY1260–62	pY1276	pY1289	pY1328
PI3KR1 N		*	*		**		*	**	
PI3KR1 C		*						***	
PI3KR1 NC		**	**	**	***	*	***	***	
PI3KR2 N		*	**		**	**	**	***	
PI3KR2 C							*		
PI3KR2 NC		***	**	**	***	*	***	***	
PI3KR3 N		*	**	*	**		**	***	
PI3KR3 C		**		**	**		**	***	
PI3KR3 NC		***	*	***	**	***	***	***	
ABL1								*	
ABL2	***	**		**	**		**	***	*
RASA1N	*		*	*	*		*		
GRB7			***		*		*		
SYK NC	*			*			*		
HCK							*	**	
PLCG1 C							*	**	
PLCG1 NC							*	**	
SHC1							*	*	
SHC3							*	**	
CRK							**		
NCK1							**		
NCK2							*		
CRKL							**		
JAK2							*		
VAV2							**		
VAV1								**	
SRC								*	
TENS1								*	
DAPP1								*	

Domain source	pY868	pY1054	pY1197	pY1222	pY1260	pY1260–62	pY1276	pY1289	pY1328
FER								**	
ITK								**	
LYN								**	
PTK6							**		
TENC1				*					
SHC1-PTB									**

Data summarized from Jones *et al.*,[133] Supplementary Table 4. Lower affinity interactions detected in the study may also be seen in this Table. For proteins with two SH2 domains, these were tested separately (N, C) or together (NC).

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Table 4

Interesting proteins with cytoplasmic interactions with ERBB3

	References
Some proteins interacting at ERBB3 phospho-tyrosine sites[a]	
Phosphatidyl inositol 3-kinase, p85 regulatory subunit (PI3KR)	59,132,133,135,136,137,139,140
GRB2/GRB7, adapter	132,133,145
c-SRC, kinase	133
SHC, adapter	59,88,132,133,146–149
Protein tyrosine kinase 6(PTK6, BRK)	125,133
Phospholipase γ1 (PLCG1), signal transducer	133,150
ABL1/2, cytoplasmic tyrosine kinases, oncogenes	133
RasGAP (RASA1N), ras proto-onogene regulator	133,151
SYK, cytoplasmic tyrosine kinase, tumor suppressor	133
CRKL, activator of ras and jun oncogenes	133
VAV1/2, oncogenes	133
Proteins interacting at ERBB3 juxtamembrane sites	
ERBB3-binding protein 1 (EBP1), transcription and protein translation regulator	154
P23, homolog of mouse transplantation antigen	174
Other interacting proteins	
BMS/ETK, nonreceptor tyrosine kinase	171
RGS4, regulator of G protein signaling	172
Early growth response protein 1 (EGR1), transcription factor	172

[a]For a complete list, see Table 3 and Ref.133

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Table 5

ERBB3 in mammary cancers and cancer cells

	References
Evidence implicating ERBB3 in mammary cancer development	
ERBB3 activation in mammary tumors in transgenic mice	188–190
Increased ERBB3 mRNA or protein in many primary human breast cancers	184,186,191,192,195,199
Clear role in survival and cell growth in many human breast cancer cell lines	143,211–214,221,228–231
Upregulation as a mode of escape from mammary tumor cell suppression by tyrosine kinase inhibitors, via pAkt feedback	127,139
Ambiguities	
Variable relationship of mRNA or protein to clinical prognosis	High ERBB3 favorable or null: 197,198,200
	High ERBB3 unfavorable: 184,186, 193–195,200,201,203
Conflicting evidence regarding relationship to estrogen receptor expression	193,200,203
NRG-dependent mammary cell differentiation, apoptosis, or growth suppression	82,184,232,233

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Table 6

Interesting features of ERBB3 in ovarian and prostate cancer

	References
Ovarian cancer	
ERBB3 expression usually high and associated with poor prognosis	237–246
Possible ERBB3 activation not involving other ERBB receptors	128,247
Truncated ERBB3 transcripts and proteins, including secreted forms	23
Prostate cancer	
Consistent ERBB3 overexpression and association with poor prognosis	79,251–256
Growth suppression by release of ERBB3-bound EBP1, an androgen receptor inhibitor, after NRG treatment of androgen responsive cells	162–164
Enhancement of androgen receptor phosphorylation and stability and actions, as ERBB2/ERBB3 complex, independent of EGFR and AKT	248
Nuclear localization of ERBB3 variably associated with hormone dependence and with microenvironment	79,274
Secreted ERBB3 isoform enhancing bone invasion	26,275,276

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Table 7

Evidence for involvement of ERBB3 in gastrointestinal cancers

	References
Colorectal cancers	
Variable association of mRNA and protein levels with poor prognosis	320,325–332
Reduced nuclear ERBB3 in colon tumors, especially carcinomas	332
High expression in colorectal cancer cell lines, association with invasiveness	141,333–335
Pancreatic cancers	
Consistent upregulation of mRNA and protein and association with poor prognosis	334,336–339
Correlation with radio resistance	340
Gastric cancer	
Increased expression in cancers	277,341,342
High expression in gastric cancer cell lines, and secretion of a truncated product	22
NRG activation of both EGFR/ERBB3 and ERBB2/ERBB3 heterodimers; increase in motility	321,343
Reduced differentiation and increased motility in response to NRG	344
Head and neck cancers (oral cavity)	
Highly expressed and associated with poor prognosis of oral cancers in most though not all studies	Positive:[347–351] No link:[352,353]
Correlation of expression with resistance to EGFR inhibitor gefitinib	354
Increase in carcinogen-induced oral carcinomas in rats	357

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Table 8

Involvement and characterization of ERBB3 in lung cancer

	References
Correlation of expression with poor prognosis	374–378
Lung tumorigenesis in transgenic mice	379,380
ERBB3-dependent signaling pathways leading to proliferation, survival and invasiveness of lung adenocarcinoma cells in culture and as xenografts	139,140
High expression in lung cancer and cancer cell lines relative to responsiveness to therapeutic effects of EGFR inhibitors	77,380,392,416
Stimulation by NRG/ERBB3 pathway	153,388
Correlation of NRG expression with insensitivity to EGFR inhibitor	425,426
Activation of ERBB3 by the MET receptor in lung cancer cells developing resistance to EGFR inhibitor	121,122
Importance of AKT activation	140,388,389,391–394

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Table 9

Effects of *in vivo* siRNA treatment on growth of lung adenocarcinoma A549 cells as xenografts

	Average tumor size (percent of average size in untreated controls)		
	Exp 1	**Exp 2**	**Exp 3**
ERBB3 siRNA	40.9 ± 17.7	20.6 ± 4.5	33.9 ± 5.5
	$P = 0.029$	$P<0.0001$	$P<0.0001$
AKT2 siRNA	34.2 ± 7.6	21.6 ± 3.5	46.2 ± 13.2
	$P = 0.0003$	$P<0.0001$	$P = 0.0028$
Nonsilencing siRNA	ND	87.1 ± 22.2	90.0 ± 17.6
		$P = 0.57$	$P = 0.58$
Saline only	ND	79.7 ± 22.1	ND
		$P = 0.38$	

A549 cells (5×10^6) from a proliferating culture were implanted subcutaneously into female Swiss athymic nude mice. When the tumors reached a size of 2 × 2 to 2.5 × 2.5mm (2–2.5 weeks after implantation), the mice were injected intravenously through the tail vein with 2 μg g^{-1} body weight of saline solutions of *ERBB3* siRNA, *AKT2* siRNA, nonsilencing siRNA, saline, or nothing, 5 days per week for 3 weeks. Sequences of the siRNAs have been reported previously.[140] Tumors were measured weekly. In each treatment group there were 4–6 mice (Exp 1) or 10–12 mice (Exps 2 and 3). After 3 weeks, the average size of the tumors in the untreated group was determined, and each tumor in the treated mice was measured and its size expressed as a percent of the untreated average. Results in the table are averages of these sizes ± s.e. All of the data sets were found to be normally distributed; the *P*-values are based on one-sample *t*-tests. ND, not done. From Sithanandam *et al*., in preparation.

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Table 10

Possible approaches for therapeutic targeting of ERBB3

	References
Experimental approaches under study	
RNA aptamers to extracellular domain (breast cancer cells)	431
Synthetic designer zinc finger transcription factor (squamous carcinoma cells)	432
Vitamin E isomer (breast cancer cells)	433
Micro-RNA downregulation of mRNA (breast cancer cells)	434
siRNA downregulation of mRNA (lung cancer cells)	Table 9
Suggested approaches	
NRDP1 ubiquitin ligase as negative regulator	435
Blockage of transactivation	436
Blockage of NRG nucleocytoplasmic trafficking	437
Specific inhibitor of ADAM17 sheddase	425,426