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ErbB-β-Catenin Complexes Are Associated with Human Infiltrating Ductal Breast and Murine Mammary Tumor Virus (MMTV)-Wnt-1 and MMTV-c-Neu Transgenic Carcinomas*

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Simultaneous deregulation of both Wnt and ErbB growth factors has previously been shown to result in the cooperative induction of mammary gland tumors. Using the murine mammary tumor virus (MMTV)-Wnt-1 transgenic model of mammary carcinoma, we have identified an unvarying association between β-catenin and epidermal growth factor receptor/c-Neu (ErbB1/ErbB2) heterodimers in mammary gland tumors, indicating a requirement for ErbB signaling in Wnt-mediated tumorigenesis. Expansion of these observations to a second transgenic model, MMTV-c-Neu, demonstrated similar tumor-specific interactions, including an ErbB1 ligand-inducible phosphorylation of both β-catenin and c-Neu. Direct relevance of these findings to human breast cancer was established upon examination of a set of human infiltrating ductal breast adenocarcinoma and lymph node metastasis tissues taken at surgery. These data revealed increased levels of β-catenin in tumors and metastases *versus* normal breast as well as an association between β-catenin and c-Neu that measurably occurs only in neoplasia, most strongly in metastatic lesions. These studies have identified a seemingly indispensable interaction between β-catenin and epidermal growth factor receptor/c-Neu heterodimers in Wnt-1-mediated breast tumorigenesis that may indicate a fundamental signaling event in human metastatic progression.

The ErbB family of transmembrane receptor tyrosine kinases (ErbB1 or epidermal growth factor receptor (EGFR),[1] ErbB2 or Her2/c-Neu, ErbB3, and ErbB4) and ligands (including EGF-like ligands and neuregulin-like ligands) have been consistently implicated in mammary gland tumorigenesis in both humans and rodents (1–3). The ErbB receptors are activated by the binding of cognate ligands, resulting in homo- and heterodimer receptor formation and subsequent phosphorylation of a wide variety of cellular substrates (*i.e.* mitogen-activated protein kinase, signal transducers and activators of transcription, phospholipase Cγ, c-*src*, etc. (4)), most often leading to increased cellular mitogenesis. The development of transgenic lines targeting overexpression of either the ligands (3) or the receptors (5, 6) to the mammary gland invariably results in tumorigenesis and has been useful in elucidating the involvement of other proteins in ErbB-related oncogenesis, such as c-Myc (7) and cyclin D1 (8). One such study utilized insertional mutagenesis of the WAP-transforming growth factor α (TGFα) model to identify genes able to synergize with EGFR activation in tumor progression (9). These studies resulted in the observation that Wnt-1 and Wnt-3 are up-regulated in this model of ErbB-induced tumorigenesis, indicating that these pathways may cooperate during transformation.

The Wnts are a family of secreted proteins whose overexpression results in the accumulation of β-catenin, a protein involved in both cellular adhesion (as a critical component of the E-cadherin-actin complex found at adherens junctions) and oncogenesis (10, 11). Binding of the soluble Wnt protein to the Frizzled receptor results in the inactivation of glycogen synthase kinase-3β, which normally phosphorylates excess β-catenin and causes its ubiquitination and degradation (10, 12). Wnt signaling-induced inactivation of glycogen synthase kinase-3β results in the loss of this regulating mechanism, causing an accumulation of β-catenin. This increased pool of β-catenin can translocate to the nucleus, where it functions as a transcriptional coactivator of LEF/TCF transcription factors, resulting in the transcription of the protooncogenes c-*myc* and cyclin D1, among others (13–15). Importantly, *in vitro* studies have shown that the ErbB receptors can induce a tyrosine phosphorylation of β-catenin (16, 17), which can prevent the binding of β-catenin to E-cadherin, potentially shifting β-catenin to the oncogenic pathway (18). In fact, tyrosine phosphorylation of β-catenin can result in enhanced invasion and metastasis in breast cancer and melanoma cell lines (16, 17). These studies indicate that the ErbB receptor kinase family and β-catenin may be cooperating in tumorigenesis.

The creation of MMTV-Wnt-1 (19, 20) and MMTV-β-cateninΔN (β-catenin with increased stability due to a lack of glycogen synthase kinase-3β phosphorylation residues) (21) transgenic mice resulted in similar phenotypes, including the stochastic development of unifocal, nonmetastatic mammary gland adenocarcinomas. Additionally, MMTV-β-cateninΔN transgenics display increased expression of c-*myc* and cyclin D1 (21). These studies indicate that the mechanism of Wnt-induced mammary carcinoma is indeed through the accumulation of β-catenin. Recent evidence suggests that β-catenin may play a pivotal role in the development and diagnosis of human breast cancer (22). Translocation of β-catenin from the adherens junction to the cytoplasm and nucleus correlates signifi-

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; MMTV, mouse mammary tumor virus; TGFα, transforming growth factor α; WAP, whey acidic protein.

cantly with poor patient outcome (22). Interestingly, this shift in localization is commonly observed in a number of human cell lines following tyrosine phosphorylation of β-catenin (16, 17, 23, 24).

We have investigated the relationship between ErbB receptor kinases and β-catenin during mammary gland tumorigenesis utilizing human adenocarcinoma samples as well as the MMTV-Wnt-1 (19) and MMTV-c-Neu (5) transgenic models. We have identified an interaction between the ErbB receptors and β-catenin that is specific to mammary gland tumors in transgenic mice, and, importantly, we have found this interaction reiterated in human breast cancer. These studies identify an important interaction between β-catenin and the ErbB family of receptors in breast tumorigenesis that may indicate a fundamental signaling event in human metastatic progression.

MATERIALS AND METHODS

Animals—MMTV-Wnt-1 mice (FVB) were purchased from Jackson Laboratories and bred with wild type FVB females (19). MMTV-c-Neu mice (line 202) were a kind gift from W. Muller (5). All animals were housed at four mice per cage, and results were similar with virgin and pregnant females.

Lysate Preparation—Protein lysates from transgenic mice were prepared as described previously (25). For human tissue collection, primary breast tumors, axial lymph node tumors, and normal breast samples were provided by the Mayo Clinic Hospital Pathology Laboratory as discard from surgical lumpectomy or mastectomy (with Institutional Review Board approval). Tissue pathology was determined by Mayo Clinic Hospital pathologists and either fixed in methacarn or homogenized in lysis buffer immediately (25).

Immunoprecipitation and Immunoblotting—Immunoprecipitations and immunoblotting was performed as described previously (25). Immunoblot chemiluminescence was performed using Pierce Super Signal substrate; West Pico chemiluminescent substrate (midpicogram range detection) for ErbB, β-catenin, and exogenous phosphorylation; and West Dura extended duration chemiluminescent substrate (femtogram range detection) for endogenous phosphorylation detection. Antibodies used for immunoprecipitation studies are as follows: β-catenin, EGFR (1005), and c-Neu (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibodies used for immunoblotting are as follows: anti-EGFR (1005, Santa Cruz Biotechnology), anti-EGFR (ERCT, H. S. Earp, University of North Carolina, Chapel Hill, NC); β-catenin (rabbit) and c-Neu (both from Santa Cruz Biotechnology); and anti-phosphotyrosine (Transduction Laboratories (RC20-HRP) or Upstate Biotechnology, Inc. (Lake Placid, NY, G410-HRP).

Immunofluorescence—Sections were treated and analyzed as previously described (25). The following antibodies were used: Alexa anti-rabbit 488 and Alexa anti-mouse 546 (Molecular Probes, Inc., Eugene OR). Dilutions for the antibodies were as follows: EGFR (1005), 1:100; mouse-anti-β-catenin (Transduction Laboratories), 1:300; and c-Neu (Santa Cruz Biotechnology, mouse tissues, 1:250 or Neomarkers Ab17, human tissues 1:200).

RESULTS

β-Catenin Is Tyrosine-phosphorylated in MMTV-Wnt-1 Tumors—EGFR-induced tumor formation in the mammary gland is promoted by cooperative events with Wnt-mediated signaling, since both Wnt-1 and Wnt-3 were found to be up-regulated in tumor samples from WAP-TGFα transgenic animals (9). To directly examine the role of EGFR signaling in Wnt-1-mediated tumorigenesis, we utilized the MMTV-Wnt-1 transgenic mouse (19). In this transgenic model, overexpression of the soluble Wnt-1 glycoprotein results in the accumulation of β-catenin in the mammary gland, leading to hyperplasia and the stochastic formation of nonmetastatic, unifocal adenocarcinoma (20). Separate studies have demonstrated that the transforming event is probably due solely to this accumulation of β-catenin (21). Analysis of the β-catenin protein in the hyperplastic mammary glands (all nontumor mammary glands in this model are hyperplastic but will hereafter be referred to as normal) and corresponding tumors of MMTV-Wnt-1 mice revealed two species of β-catenin protein in the normal mammary gland lysates

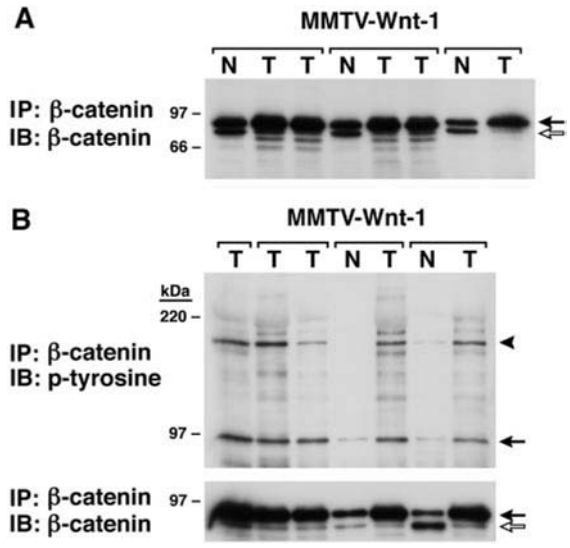

FIG. 1. **β-Catenin is tyrosine-phosphorylated in tumors from MMTV-Wnt-1 mammary glands.** *A*, protein lysates (1 mg) from both normal (*N*) and mammary gland tumors (*T*) were immunoprecipitated (*IP*) with goat anti-β-catenin and immunoblotted (*IB*) with rabbit-anti-β-catenin. *B*, immunoprecipitations were performed as described for *A* and immunoblotted with anti-phosphotyrosine antibody (RC20-HRP). Endogenous phosphorylation was detected using West Dura extended duration chemiluminescent substrate and exposed for 30 s. Normal mammary glands and tumors from a single animal are *bracketed*, and the two species of β-catenin are indicated by *arrows* (~97 kDa (*closed arrow*) and ~95 kDa (*open arrow*)).

of ~95 and 97 kDa, respectively. Interestingly, mainly the larger form (with retarded mobility in the SDS-PAGE) was observed in the tumors (Fig. 1*A*).

To determine the tyrosine phosphorylation status of β-catenin, we analyzed a set of tumor and mammary gland lysates using an enhanced anti-phosphotyrosine antibody (RC20-HRP; see "Materials and Methods") and a highly sensitive chemiluminescent substrate (West Dura extended duration; see "Materials and Methods"), capable of detecting the low level of endogenous phosphorylation present in whole tissue lysates. We detected a single phosphoprotein that comigrated with the larger form of β-catenin in both the tumor and mammary gland (Fig. 1*B*, *closed arrow*). Since the major species identified in the tumors was the larger phosphorylated form, this indicated that a kinase was preferentially associated with β-catenin in the tumors, as opposed to the normal gland. Interestingly, we detected a phosphoprotein of ~180 kDa coimmunoprecipitating with β-catenin (Fig. 1*B*, *arrowhead*), which is the size range of the ErbB receptor kinases.

EGFR and c-neu Associate with β-Catenin in Tumors but Not Normal Mammary Glands—Previous studies have demonstrated a functional relationship between β-catenin and EGFR in tumor progression (9, 16). We therefore analyzed our tumor and mammary gland samples to determine if the 180-kDa kinase we had detected immunoprecipitating with β-catenin (Fig. 1) was in fact EGFR. Upon analysis of more than 25 independent mammary gland tumors from MMTV-Wnt-1 transgenics, we found that EGFR and β-catenin interacted in 100% of the tumors (Fig. 2). Conversely, we detected minimal amounts of β-catenin coimmunoprecipitating with EGFR in the normal mammary glands taken from the same animal as the tumor (Fig. 2). Through analysis of the endogenous kinase activity of EGFR as determined by EGFR tyrosine phosphorylation, we detected variable phosphorylation of the EGFR in tumors where EGFR and β-catenin were interacting (Fig. 2*B*, *top panel*). This indicated that β-catenin phosphorylation (Fig. 1*B*) was not due solely to EGFR kinase activity and may be the

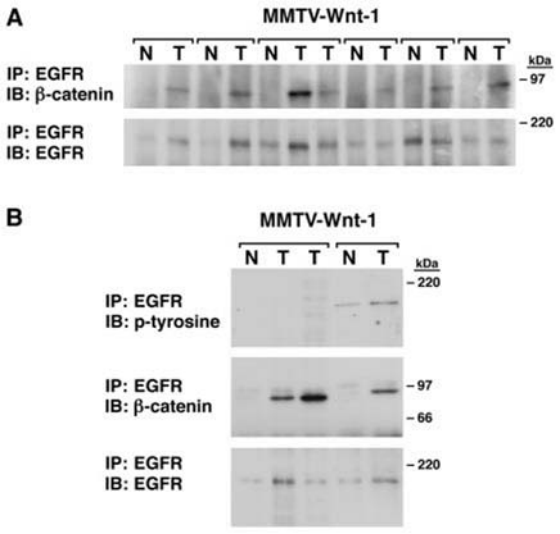

FIG. 2. **β-Catenin associates with EGFR in MMTV-Wnt-1 tumors.** *A*, protein lysates (1 mg) from both normal (*N*) and mammary gland tumors (*T*) were immunoprecipitated (*IP*) with anti-EGFR (1005) and immunoblotted (*IB*) with either rabbit-anti-β-catenin (*top panel*) or anti-EGFR antibody (ERCT) (*bottom panel*). *B*, immunoprecipitations and immunoblots were performed as described for *A* (*middle* and *bottom panels*) and immunoblotted with anti-phosphotyrosine antibody (RC20-HRP) (*top panel*). Endogenous phosphorylation was detected using West Dura extended duration chemiluminescent substrate and exposed for 30 s. Normal mammary glands and tumors from a single animal are *bracketed*.

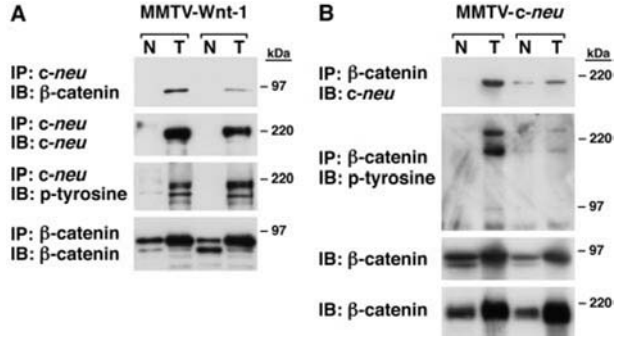

FIG. 3. **β-Catenin-EGFR-c-Neu complex formation in MMTV-Wnt-1 and MMTV-c-Neu tumors.** *A*, MMTV-Wnt-1 protein lysates (1 mg) from both normal (*N*) and mammary gland tumors (*T*) were immunoprecipitated (*IP*) with either anti-c-Neu antibody (*top three panels*) (Santa Cruz Biotechnology) or goat-anti-β-catenin (*bottom panel*). Membranes were immunoblotted (*IB*) with either rabbit anti-β-catenin (*top* and *bottom panels*), anti-c-Neu antibody (Santa Cruz Biotechnology) (*middle top panel*), or anti-phosphotyrosine (RC20-HRP) (*middle bottom panel*). *B*, immunoprecipitations (1 mg) and immunoblotting for MMTV-c-Neu transgenic animals were performed as described for *A*. Endogenous phosphorylation was detected using West Dura extended duration chemiluminescent substrate and exposed for 30 s and 1 min, respectively. Normal mammary glands and tumors from a single animal are *bracketed*.

result of an interaction with one or more of the other ErbB members. We investigated the interaction between β-catenin and c-Neu (ErbB2/Her2) in MMTV-Wnt-1 transgenics, since *in vitro* studies have shown that c-Neu and β-catenin associate in stomach adenocarcinoma cell lines (24, 26). Similar to that observed with EGFR, we found a complete concordance of c-Neu interacting with β-catenin in all tumors. In contrast, c-Neu/β-catenin associations could not be detected in the normal MMTV-Wnt-1 mammary glands (Fig. 3*A*). Importantly, almost no c-Neu expression was detected in the normal glands, indicating a dramatic up-regulation of c-Neu in the tumors.

To determine whether the interaction between the ErbB receptors and β-catenin is a common mechanism or unique to the MMTV-Wnt-1 model, we investigated a second established model of mammary carcinoma, MMTV-c-Neu (5). In this model, overexpression of unactivated rat ErbB2/c-Neu protein results in the stochastic formation of metastatic (pulmonary), unifocal mammary gland adenocarcinomas (5). Analysis of this model revealed that while complex formation occurred between phosphorylated c-Neu and phosphorylated β-catenin in MMTV-c-Neu tumors, only ~50% of the samples displayed the interaction (5 of 11) (Fig. 3*B* and data not shown). Therefore, whereas Wnt-1/β-catenin-mediated tumorigenesis always appears to involve ErbB receptors, ErbB-induced tumorigenesis apparently does not always involve β-catenin. Interestingly, all five of the MMTV-c-Neu mice displaying β-catenin-c-Neu complexes in the tumors also had pulmonary metastases, while only two of six of the negative tumors did. This may indicate a correlation between β-catenin-ErbB complex formation and metastasis.

We also detected interactions between ErbB3 and ErbB4 and β-catenin in the MMTV-Wnt-1 mammary glands but only in those tumors where exceptionally high levels of β-catenin were present, since overall levels of both ErbB3 and ErbB4 were low in both the tumors and the normal glands (data not shown). Perhaps the interaction between β-catenin and the ErbB receptors is hierarchical in nature, and the preferred interaction is with EGFR/c-Neu heterodimers and next with ErbB3 and ErbB4, as has been previously proposed regarding receptor heterodimerization (27, 28).

EGFR/c-neu Heterodimers Induce β-Catenin Phosphorylation in MMTV-Wnt-1 and MMTV-c-neu Mammary Tumors—To verify that the tyrosine phosphorylation of β-catenin was due to EGFR kinase activity, we injected tumor-bearing mice with the following EGFR-specific ligands: receptor grade epidermal growth factor (EGF), TGFα, or amphiregulin. It has been previously shown that EGF injection into mice induces EGFR kinase activation as well as transphosphorylation of dimerizing ErbB partners and subsequent phosphorylation of interacting proteins (25, 28). All three ligands induced tyrosine phosphorylation of β-catenin in tumors from both MMTV-Wnt-1 and MMTV-c-Neu transgenic animals (Fig. 4*A*). In contrast, only minimal phosphorylation was observed in the normal gland during exogenous ligand stimulation. It should be noted that the analysis of protein phosphorylation from animals injected with EGFR ligands was performed with West Pico chemiluminescent substrate (see "Materials and Methods"), a detection method not sensitive enough to detect endogenous phosphorylation (as it was utilized in these studies). This dramatic induction of β-catenin phosphorylation detected in tumors from both MMTV-Wnt-1 and MMTV-c-Neu transgenic animals provides further evidence that the interaction between EGFR/c-Neu and β-catenin is tumor-specific.

To determine whether the phosphorylation of c-Neu and its association with β-catenin was due to a heterodimer formation with EGFR, similar transphosphorylation experiments were performed using EGF injections in both MMTV-Wnt-1 and MMTV-c-Neu mice. Injection of the cognate ligand for EGFR resulted in the phosphorylation of c-Neu in addition to EGFR, indicating heterodimer formation between these two receptor kinases (Fig. 4*B*). Note that treatment with EGF resulted in greatly increased phosphorylation of both EGFR and c-Neu receptor compared with endogenous phosphorylation of these receptors, since endogenous phosphorylation was undetectable using the Pico chemiluminescent substrate (see Figs. 1–3). Collectively, these data demonstrate that an EGFR/c-Neu heterodimer induces β-catenin phosphorylation specifically in tumors. Despite the fact that MMTV-Wnt-1 normal mammary glands are hyperplastic, EGF-induced phosphorylation of β-catenin is difficult to detect in those tissues, suggesting this

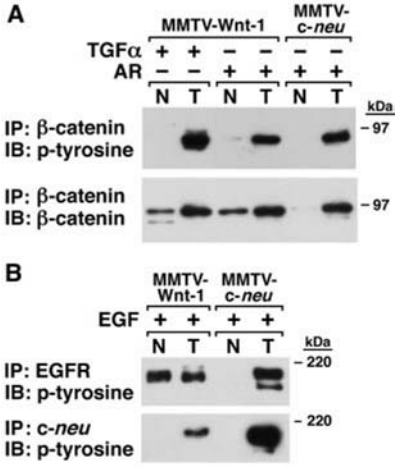

FIG. 4. **β-Catenin is phosphorylated by EGFR-specific ligands in both MMTV-Wnt-1 and MMTV-c-Neu mammary gland tumors.** *A*, animals were injected with 0.8 μg/g body weight TGFα or amphiregulin, and normal (*N*) and mammary gland tumors (*T*) were immunoprecipitated (*IP*) (1 mg) with anti-β-catenin antibodies (goat). Immunoblotting (*IB*) was performed using anti-phosphotyrosine antibodies (RC20-HRP) (*top panel*) or rabbit anti-β-catenin (*bottom panel*). Exogenous phosphorylation was detected using West Pico chemiluminescent substrate and exposed for 2 min. *B*, MMTV-Wnt-1 and MMTV-c-neu protein lysates (1 mg) from both normal (*N*) and mammary gland tumors (*T*) were immunoprecipitated with either anti-EGFR antibodies (1005) (*top panel*) or anti-c-Neu antibodies (*bottom panel*) and immunoblotted with anti-phosphotyrosine antibody (RC20-HRP). Animals were injected with 1 μg/g body weight EGF. Exogenous phosphorylation was detected using West Pico chemiluminescent substrate for 45 s. Normal mammary glands and tumors from a single animal are *bracketed*.

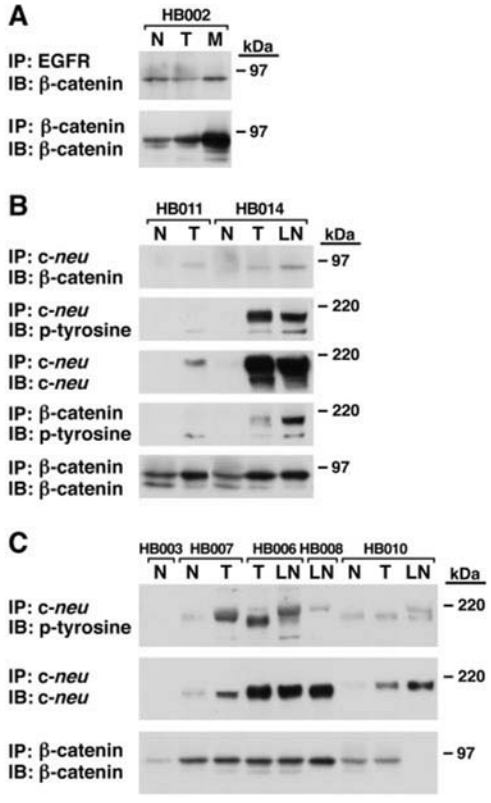

FIG. 5. **c-Neu is phosphorylated and associates with β-catenin in invasive human breast cancer.** *A*, protein lysates from normal breast (*N*), primary breast adenocarcinoma (*T*), and axillary lymph node metastasis (*M*) (2 mg) were immunoprecipitated (*IP*) with either goat anti-β-catenin (*bottom*) or rabbit anti-EGFR (*top*) (Santa Cruz Biotechnology) and immunoblotted (*IB*) with rabbit anti-β-catenin. *B* and *C*, samples described for *A* were immunoprecipitated with either rabbit-anti-c-neu (Santa Cruz Biotechnology) or goat-anti-β-catenin (Santa Cruz Biotechnology) antibodies and immunoblotted with rabbit-anti-β-catenin (Santa Cruz Biotechnology), anti-phosphosphotyrosine (Transduction Laboratories), or mouse anti-c-Neu (Neomarkers) antibodies. Human breast (*HB*) sample numbers are denoted *above* the *brackets* (*brackets* indicate one patient) and correspond to Table I.

signaling complex is important primarily in carcinoma. Furthermore, these findings indicate that EGFR and c-Neu are constitutive components of Wnt-1-mediated tumorigenesis.

c-neu and β-Catenin Associate in Human Breast Cancer—To determine the frequency of these interactions in human breast cancer, we analyzed normal breast, breast-infiltrating ductal adenocarcinomas, and axillary lymph node metastases for overall β-catenin expression and the frequency of interactions occurring between β-catenin and members of the ErbB receptor family. Tissues were prepared directly after surgery from surgical pathology samples obtained during lumpectomy or mastectomy, and lysates were produced. Immunoblot analysis revealed increasing amounts of β-catenin protein with advancing levels of invasiveness, although in two of four samples analyzed, levels were similar in both the tumor and metastasis (Fig. 5, *A* and *B*, *bottom panels*). Our attempts to determine levels of β-catenin phosphorylation levels were unsuccessful, largely due to nonspecific contamination that masked proteins around 97 kDa in size, including β-catenin (although different anti-phosphotyrosine antibodies were attempted). Fortunately, we were able to detect tyrosine-phosphorylated proteins of ~180 kDa coimmunoprecipitating with β-catenin in the metastatic samples (Fig. 5*B*, *fourth panel*, and data not shown). Analysis of EGFR-β-catenin coimmunoprecipitation complexes revealed that interactions between the two proteins occur in all samples, with variable increases in the tumors and metastases (Fig. 5*A*, and data not shown; total samples analyzed: metastases, $n = 4$; tumors, $n = 5$; and normal, $n = 5$). Since the expression of EGFR itself was highly variable from patient to patient and was frequently highest in the normal breast (data not shown), it is difficult to ascertain whether interactions between β-catenin and EGFR were higher in one tissue type over another.

Upon examination of c-Neu protein, we commonly observed an increase in expression in the primary and metastatic tumor *versus* the normal breast (Fig. 5, *B* and *C*). Examination of c-Neu immunoprecipitations revealed β-catenin association in the primary tumor and metastasis, with the strongest interaction occurring in the metastatic lesion (Fig. 5*B*, total samples analyzed: metastases, $n = 5$; tumors, $n = 8$; and normal, $n = 10$). This observation was true even in patients where c-Neu and β-catenin expression levels were equal in both tumor and lymph node (Fig. 5, *B* and *C*, samples HB014 and HB006 and data not shown). These data indicate that c-Neu is the ErbB kinase specifically complexed with β-catenin in invasive human breast cancer. As the interaction between c-Neu and β-catenin in the MMTV-c-Neu mice occurs only in those animals with pulmonary metastases (see above section), a compelling argument can be made for these protein interactions correlating with invasive disease.

It is interesting to note also that the majority of our patient samples (9 of 10) were graded pathologically as HER2/Neu-negative (Table I). The primary tumors from each patient were graded using the DAKO HercepTest and graded 0 or 1+ (only 2+ or 3+ is considered positive for HER2/Neu expression), whereas immunoprecipitation of c-Neu and immunoblotting allowed for readily detectable HER2/Neu protein expression in most (5 of 9) of these samples (Fig. 5*C* and data not shown). Importantly, analysis of endogenous phosphorylation of the c-Neu protein present in these tumors demonstrated activated receptor, indicating that the protein present is in fact func-

TABLE I
Patient characteristics

ID no.	Stage at diagnosis	Stage at surgery	Patient age at surgery	Modified Bloom-Richardson	Type	HER2/*neu*	Estrogen receptor/ progesterone receptor	Lymph nodes obtained for study	Metastatic carcinoma
HB001[a]	2	2B	41	3	Infiltrating ductal carcinoma	Negative	Positive/negative	Yes	8 of 23 lymph nodes
HB002	3	3	51	3	Infiltrating ductal carcinoma	Negative	Negative	Yes	7 of 7 lymph nodes
HB003[b]	2	2	85	1	Infiltrating ductal carcinoma	Negative	Positive	No	1 of 14 lymph nodes
HB005[c]	4	4	54	3	Infiltrating ductal carcinoma	Negative	Negative	Yes	7 of 7 lymph nodes lung, liver
HB006	2	2	58	2	Infiltrating ductal carcinoma	Negative	Positive	Yes	1 of 14 lymph nodes
HB007	1	1	69	3	Infiltrating ductal carcinoma	Negative	Negative	No	Negative
HB008	2	2	70	3	Infiltrating ductal carcinoma	Negative	Positive	Yes	4 of 16 lymph nodes
HB009[c]	2	2	52	2	Infiltrating ductal carcinoma	Negative	Negative	No	0 of 21 lymph nodes
HB010[d]	4	4	68	3	Infiltrating lobular carcinoma	Negative	Positive/negative	Yes	9 of 16 lymph nodes
HB011	2	2	59	2	Infiltrating lobular carcinoma	Negative	Positive	No	0 of 13 lymph nodes
HB014	3	3a	77	3	Infiltrating ductal carcinoma	Positive	Positive/negative	Yes	8 of 16 lymph nodes

[a] See Fig. 6.
[b] Lymph node not available for study.
[c] Molecular data not shown.
[d] No β-catenin expression in lymph node.

tional (Fig. 5*C*, *top panel*). Further, in each clinically HER2/Neu-negative case with an associated lymph node metastasis ($n = 4$), the c-Neu in the metastatic lesion is hyperphosphorylated compared with the primary tumor, as can be ascertained by the increased retardation of the phosphorylated species in the acrylamide gel (Fig. 5*C*, *top panel*, *LN*). This may indicate an increased activity in the metastasis *versus* the primary tumor. It is interesting to note that in our single HER2/Neu clinically positive patient (HB014), both the primary tumor and the lymph node metastasis were hyperphosphorylated.

EGFR and c-neu Colocalize with β-Catenin in Both Membrane and Cytoplasmic Compartments of Breast Tumors–Previous reports suggest that cytoplasmic/nuclear β-catenin expression is an "activated" phenotype and correlates with a poor patient prognosis (22). To determine the site of β-catenin/ErbB interactions in our patient samples, we used confocal microscopy to analyze both tumors and normal glands. In human and MMTV-Wnt-1 breast tumors, β-catenin was detected in the membrane and cytoplasm, indicating a similarity between the transgenic model and human disease. Substantial colocalization of the ErbB receptors with β-catenin was observed in human, MMTV-Wnt-1, and MMTV-c-Neu primary tumors and human lymph node metastases. Colocalization of both EGFR and c-Neu with β-catenin in the tumor samples from MMTV-c-Neu appeared to occur in close proximity to the membrane (Fig. 6, *F* and *H*), whereas human and MMTV-Wnt-1 colocalization was present throughout the cytoplasm (Fig. 6, *A*, *B*, and *D*). In contrast, very limited colocalization was detected in the normal mammary glands (Fig. 6, *C* and *G*), with the exception of EGFR/β-catenin in MMTV-c-Neu, where colocalization was observed in some of the normal glands (Fig. 6*E*). These data demonstrate that interactions between β-catenin and EGFR and c-Neu do occur in the cytoplasm, similar to where β-catenin is found to be localized in the activated phenotype observed by other groups (22).

DISCUSSION

We have discovered a strikingly common association between EGFR/c-Neu and β-catenin that is highly restricted to tumors of the mouse mammary gland and results in the tumor-specific phosphorylation of β-catenin. Remarkably, these results correspond closely with human breast cancer, since c-Neu and β-catenin form a complex in invasive breast cancer. Numerous reports indicate that under certain growth conditions, both a normal mammary gland cell line (29) and carcinoma cell lines (16, 17, 23, 24) are able to respond to EGFR ligands by tyrosine phosphorylation of β-catenin. This interaction can result in a loss of adherens junction formation and an increase in cellular invasion. This is the first report to demonstrate that these interactions occur *in vivo* and are unique to the transformed breast. Importantly, through analysis of these multiple models, we can hypothesize a paradigm of ErbB-induced β-catenin phosphorylation that results in β-catenin deregulation and progression to invasive breast cancer.

The MMTV-Wnt-1 transgenic model induces the overexpression/accumulation of β-catenin in the mouse mammary gland, resulting in the formation of unifocal, stochastic adenocarcinomas (19, 20). One primary reported mechanism of β-catenin-induced tumorigenesis is translocation to the nucleus wherein β-catenin functions as a transcriptional co-factor and increases the production of such oncogenes as c-*myc* and cyclin D1 (13–15). In the four patients and the MMTV-c-Neu transgenic animals analyzed for β-catenin/c-Neu colocalization, β-catenin was not observed in the nucleus. No colocalization of β-catenin with EGFR or c-Neu was seen in the nucleus of the MMTV-Wnt-1 transgenics either but instead was observed in the cytoplasm and at the cell membrane. One previously reported

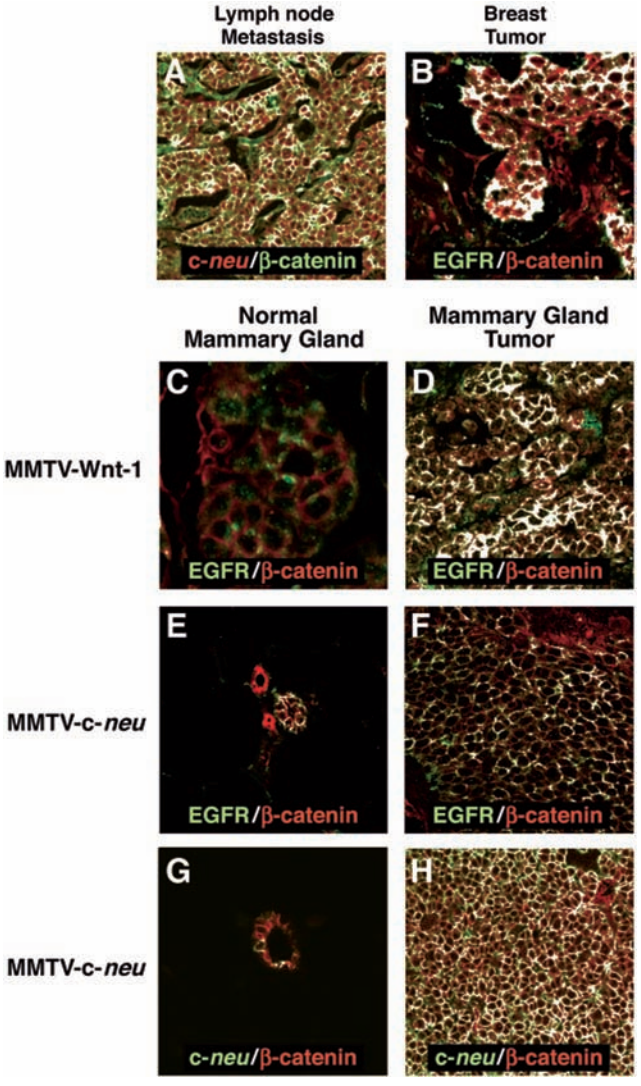

FIG. 6. **ErbB receptors colocalize with β-catenin in human and mammary carcinomas.** *A*, immunohistochemical detection of c-Neu/β-catenin complex with mouse-anti-c-Neu (Ab17)/Alexa anti-mouse 546 and rabbit-anti-β-catenin/Alexa anti-rabbit 488 (*white* denotes colocalization; overlays depicted). *B–F*, immunohistochemical detection of EGFR-β-catenin complex with rabbit anti-EGFR (1005)/Alexa anti-rabbit 488 and mouse anti-β-catenin/Alexa anti-mouse 546. *G* and *H*, immunohistochemical detection of c-Neu-β-catenin complex with rabbit anti-c-Neu (Santa Cruz Biotechnology) and Alexa anti-rabbit 488 and mouse anti-β-catenin. *A*, human infiltrating ductal carcinoma (HB001); *B*, human lymph node metastasis (HB001); *C* and *D*, MMTV-Wnt-1; *E–H*, MMTV-c-Neu. *C*, *E*, and *G*, normal mammary gland; *D*, *F*, and *H*, tumors. Sections were examined at ×400 magnification, except *B*, which was ×630.

result of the association of β-catenin with the ErbB transmembrane kinases is to induce a separation of β-catenin from E-cadherin (16). Analysis of the soluble fraction of tumor and normal lysates demonstrated no disruption of β-catenin/E-cadherin association in the MMTV-Wnt-1 transgenic tumors (data not shown). Therefore, these interactions may be occurring in the presence of intact β-catenin/E-cadherin binding, a phenomenon previously demonstrated in inflammatory breast carcinoma wherein the invasive cell line MARY-X has intact E-cadherin/β-catenin binding at the adherens junctions (30). Alternatively, the β-catenin-ErbB complex may include other, as yet unidentified, proteins, which promote tumorigenesis through β-catenin relocalization and interaction. Preliminary data in our laboratory indicate that at least one other protein may be involved in the complex, the tumor antigen MUC1 (data not shown), and these interactions are currently being pursued. In addition, the actin-bundling protein fascin both is up-regulated by ErbB2 expression and interacts with β-catenin at the cell membrane, since its overexpression can result in the induction of cell motility (31–34). Therefore, fascin may also be a potential candidate for interacting with alternatively regulated β-catenin, and these possible interactions are currently being investigated.

Interestingly, clinical analysis using the HercepTest has graded eight of nine of the primary tumors in this study HER2/Neu-negative for the purposes of adjuvant therapy (Table I; Dako HercepTest score of 0 or 1+). Conversely, immunoblot and immunofluorescent analysis revealed readily detectable levels of HER2/Neu in most (five of nine) of these same tumors, indicating that our methodology is more sensitive than what is performed clinically. Anti-HER2/Neu therapy (Trastuzumab and Herceptin, Genentech, San Francisco, CA) is focused on treating those patients whose biopsies indicate HER2/Neu overexpression. Our data may indicate that it is not merely the level of HER2/Neu or EGFR expression that is pertinent to cancer progression but also the appropriation of the function of proteins responsible for cell adhesion, such as β-catenin. Whereas most of the tumors in this study were clinically classified as Her2/Neu-negative, the c-Neu receptor in these tumor samples is clearly expressed and engaged in a potentially oncogenic activity through its heightened phosphorylation and interaction with β-catenin. It is interesting to note that treatment of a low c-Neu-expressing cell line (MCF-7) with 4D5 (the nonhumanized precursor to Trastuzumab) resulted in the inhibition of motility and invasion, encouraging the authors to speculate on a role for anti-c-Neu therapy in treatment of breast cancer with low receptor expression (35, 36). Whereas ~20–30% of patients with metastatic breast cancer clinically overexpress Her2/Neu (37), the treatment group potentially benefited by Herceptin therapy may be much larger. The targeting of c-Neu in those cancers where expression is low (and regarded as negative by standard clinical testing) could prevent ErbB-induced phosphorylation of β-catenin and the subsequent signaling events that occur in invasive disease.

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