Distinct ErbB-2–Coupled Signaling Pathways Promote Mammary Tumors with Unique Pathologic and Transcriptional Profiles

Babette Schade,¹ Sonya H.L. Lam,¹ Daniela Cernea,² Virginie Sanguin-Gendreau,¹ Robert D. Cardiff,² Boonim L. Jung,¹ Michael Hallett,² and William J. Muller¹

¹Molecular Oncology Group, McGill University Health Centre, and ²McGill Centre for Bioinformatics, McGill University, Montreal, Quebec, Canada; and ³Center for Comparative Medicine, University of California–Davis, Davis, California

Abstract
ErbB-2 overexpression and amplification occurs in 15% to 30% of human invasive breast carcinomas associated with poor clinical prognosis. Previously, we have shown that four ErbB-2/Neu tyrosine-autophosphorylation sites within the cytoplasmic tail of the receptor recruit distinct adaptor proteins and are sufficient to mediate transforming signals in vitro. Two of these sites, representing the growth factor receptor binding protein 2 (Grb2; Neu-YB) and the Src homology and collagen (Shc; Neu-YD) binding sites, can induce mammary tumorigenesis and metastasis. Here, we show that transgenic mice bearing the two other ErbB-2 autophosphorylation sites (Neu-YC and Neu-YE) develop metastatic mammary tumors. A detailed comparison of biological profiles among all Neu mutant mouse models revealed that Neu-YC, Neu-YD, and Neu-YE mammary tumors shared similar pathologic and transcriptional features. By contrast, the Neu-YB mouse model displayed a unique pathology with a high metastatic potential that correlates with a distinct transcriptional profile, including genes that promote malignant tumor progression such as metalloproteinases and chemokines. Furthermore, Neu-YB tumor epithelial cells showed abundant intracellular protein level of the chemokine CXCL12/SDF-1A, which may reflect the aggressive nature of this Neu mutant mouse model. Taken together, these findings indicate that activation of distinct Neu-coupled signaling pathways has an important impact on the biological behavior of Neu-induced tumors. [Cancer Res 2007;67(16):7579–88]

Introduction
The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases are type I transmembrane proteins that consist of four family members; the epidermal growth factor receptor (EGFR/ ErbB-1/HER1), Neu (ErbB-2/HER2), ErbB-3 (HER3), and ErbB-4 (HER4; refs. 1, 2). This family has been implicated in many types of human cancers, particularly in breast cancer (3). Indeed, ErbB-2 is amplified and overexpressed in 15% to 30% of breast cancer patients with invasive ductal breast carcinoma, which is associated with poor prognosis (4, 5). Transgenic mouse models over-expressing constitutively activated Neu mutants in the mammary epithelium develop multifocal metastatic mammary tumors. Moreover, mice overexpressing wild-type Neu develop focal mammary tumors, albeit with delayed kinetics. Further molecular analyses revealed that tumor progression in these strains is dependent on the occurrence of somatic mutations in the juxta-transmembrane region of the Neu receptor that have been shown to result in the constitutive activation of the receptor (6–10). These observations suggest that the activation of Neu is a critical event in the induction of mammary tumors.

Neu activation results in the transphosphorylation of discrete tyrosine residues within the cytoplasmic tail. These phosphorylated residues serve as docking sites for Src homology 2 and/or phosphotyrosine binding (PTB) domain-containing adaptor proteins. These adaptors regulate intracellular signaling pathways controlling gene expression of molecules that affect many biological processes like cell proliferation, differentiation, survival, angiogenesis, and invasion. We have previously shown that four tyrosine-autophosphorylation sites (Y1144/YB, Y1201/YC, Y1227/YD, Y1253/YE) within the Neu cytoplasmic tail are required to transduce Neu-transforming signals in vitro (11). Indeed, Neu mutant receptors containing only one functional autophosphorylation site showed that each tyrosine is sufficient to mediate Neu-dependent transformation in vitro. These sites bind distinct adaptor proteins that include growth factor receptor binding protein 2 (Grb2; Y1144), Src homology and collagen (Y1227), and Crk (Y1201), suggesting Neu couples to distinct effector pathways to promote transformation (11).

To dissect the biological importance of these individual signaling pathways in mammary tumorigenesis, transgenic mice expressing constitutively active Neu mutant receptors that only couple to Grb2 (Neu-YB) or Shc (Neu-YD) have been previously generated. Both mutant strains develop mammary tumors but display dramatic differences in tumor morphology, tumor burden, and metastatic potential. These observations suggest that the differential ability of Neu to couple distinct signaling pathways has a profound effect on its capacity to induce metastatic phenotypes in vivo (12). However, the underlying molecular basis for this biological outcome is poorly understood.

To further explore the biological properties of these signaling pathways in Neu-mediated mammary tumorigenesis, we generated and characterized transgenic mice overexpressing the remaining two autophosphorylation mutants (activated Neu-YC and Neu-YE binding site) in the mammary gland. Like the previously characterized Neu-YD strain (12), both transgenic strains rapidly developed multifocal mammary tumors. Moreover, a detailed comparison of pathologic and transcriptional profiles among the Neu mutant mouse models revealed that engagement of the individual autophosphorylation sites result in common features but also dramatic differences. Specifically, Neu-YC, Neu-YD, and Neu-YE mammary tumors shared striking similarities in pathologic features and gene expression patterns. Conversely, YB-induced
tumors possessed a unique transcriptional profile and pathology. Furthermore, the YB-specific expression of CXCL12/stromal-derived factor 1α (SDF-1α) correlates with its invasive tumor phenotype. Taken together, these observations suggest that differential activation of Neu-coupled signaling pathways can have a profound effect on the biological behavior of Neu-induced tumors.

Materials and Methods

Generation and identification of transgenic mice. Mouse mammary tumor virus (MMTV)/YC and MMTV/YE transgenic mice were generated as previously described (12). Founder animals were identified by Southern blot analysis using a fragment that corresponds to the SV40 polyadenylation cassette as previously described (12). Routine colony maintenance was done by PCR genotyping. Mammary tumor formation was monitored in nulliparous mice by weekly physical palpation.

Histologic analysis. Whole mount analyses were done on the left thoracic mammary gland (13). Mammary tumors and lung tissue were harvested from mice that were tumor bearing for 6 weeks. Tissue was fixed and paraffin embedded as described previously (12). Paraffin sections of 5 μm were stained with H&E (Histology Service, McGill University). Lung metastases were identified by microscopic analysis of lung sections. Immunohistochemical staining was done on mammary tumor sections as previously described (14). Sections were incubated first with a CXCL12- or CXCR4-specific antibody (MAB350, R&D Systems; ab2074, Abcam Inc.) and then with the Elite anti-mouse immunoglobulin G (IgG) Vectastain ABC kit (PK-6102, Vector Laboratories) according to manufacturer’s instructions.

Immunoprecipitation and immunoblotting. Tissue samples were prepared as previously described (8). Neu and ErbB-3 immunoprecipitations were done with 1 mg of cell lysate using the Ab4 mouse monoclonal antibody (Oncogene Research Products, Inc.) and the C17 rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc.) as described (8). Neu coimmunoprecipitations were done with 250 μg of protein lysate. Immunoblot analyses were done on 50 μg of total cell lysate as described (8, 12) using the following antibodies: Neu (C-18, Santa Cruz Biotechnology; 1:1,000), ErbB-3 (C17, Santa Cruz Biotechnology; 1:1,000), Grb2 (C23, Santa Cruz Biotechnology; 1:1,000), pTyr (PY20, BD Biosciences; 1:1,000), mitogen-activated protein kinase kinases 1 and 2 (Cell Signaling Technology; 1:1,000), and ErbB-3 (C17, Santa Cruz Biotechnology; 1:1,000).

Figure 1. MMTV-activated Neu-YC and Neu-YE mice develop mammary gland hyperplasias. A, the Neu receptors harbor an activating in-frame deletion in the extracellular cysteine-rich domain and tyrosine to phenylalanine substitutions of tyrosine-autophosphorylation sites within the cytoplasmic tail. A single tyrosine was reconstituted to restore binding of distinct adaptor molecules (Grb2/YB, Crk/YC, Shc/YD). B, whole mount analysis (top) and H&E sections (bottom) of mammary glands derived from Neu-YC and Neu-YE virgin females at 17 wks of age. All mammary whole mounts showed a certain degree of alveolar development and foci of atypical ductal proliferation. The YC mice display ductal ectasia and foci of atypical proliferating cells forming acinar structures (left). The YE females showed less duct ectasia, multiple side buds, and foci with solid-nodular proliferation (right). Morphology of normal mammary glands (FVB strain) can be obtained at http://ccm.ucdavis.edu/bcancerccd/contents.html.
protein kinase (MAPK; 9102, Cell Signaling Technology; 1:1,000), p-MAPK (9101, Cell Signaling Technology; 1:1,000), Akt (9272, Cell Signaling Technology; 1:1,000), p-Akt (9275, Cell Signaling Technology; 1:1,000), p-ErbB-2 Tyr877 (2241, Cell Signaling Technology; 1:1,000), and CXCR4 (ab2074, Abcam Inc.; 1:1,000). Horseradish peroxidase–conjugated secondary antibodies (1:2,500) were obtained from The Jackson Laboratory.

RNA extraction, linear amplification, and labeling. Total RNA was isolated from 10 individual flash-frozen mammary tumor samples derived from our MMTV/neu-ndl-NYPD, -YB, -YC, -YD, -YE, and the parental MMTV/neu NDL2-5 strains using the RNeasy Midi Kit (Qiagen). RNA quantity and quality were determined using a spectrophotometer (Nanodrop), the BioAnalyzer 2100, and the RNA 6000 Pico Assay Kit (Agilent Technologies). Two RNA pools, containing equal amount of total RNA from five individual tumors, were generated from each strain and functioned as a biological repeat. A total of 500 ng of total RNA from each pool was subjected to one round of T7 linear amplification using the Amino Allyl MessageAmp aRNA Kit (Ambion). About 10 μg of the resulting aRNA were labeled with Cy3 and Cy5 dyes (Cy Post-labeling Reactive Dye Pack; Amersham Biosciences). The quantity and quality of the resulting amplified and labeled aRNAs were determined using the Nanodrop and the Bioanalyzer 2100 as described above.

Microarray hybridization. A total of 750 ng of labeled aRNA were hybridized onto Agilent Whole Mouse Genome Oligo Microarray Kit (G4122A; 44K) using the Agilent In situ Hybridization Plus Kit Large for 17 h at 65 °C (Agilent Technologies). Microarrays were washed according to the manufacturer’s protocol (Agilent microarray processing protocol). Samples were hybridized against a Universal Mouse Reference RNA (Stratagene). Duplicate hybridizations were done for all samples using reverse-dye labeling.

Data preprocessing and normalization. Microarray slides were scanned using a Microarray Scanner (Model G2565BA, Agilent Technologies) at 10-μm resolution according to the manufacturer’s protocol (manual ID G4180-90030). The resulting 16-bit TIFF files were quantified using the default parameters of the Feature Extraction Software (v.7.11, Agilent Technologies). An average raw signal intensity of 1500 was required in each channel, and a signal-to-noise ratio above 30 per channel. Replicates were required to have a concordance above 0.9. Raw Cy5 and Cy3 intensities were imported into BioConductor (v.1.8; ref. 15). Data preprocessing and normalization were automated using the BIAS system (16). Raw feature intensities were background corrected using the RMA background correction algorithm (17). The resulting expression estimates were

Figure 2. MMTV/Neu-YC and MMTV/Neu-YE mice develop mammary tumors with similar kinetics. A, kinetics of mammary tumor formation in Neu-YC and Neu-YE transgenic mice. Age of tumor onset indicates the time at which a mammary tumor was first palpable. t50, age at which 50% of the females first developed a tumor. n, number of mice analyzed for each strain. Δ, Neu-YC; ●, Neu-YE4; ○, Neu-YE7. B, H&E-stained sections of mammary tumors derived from virgin females expressing the Neu-YC (left) and Neu-YE (right) transgenes. The tumors displayed well-differentiated glandular patterns and were composed of cells with small, oval to round nuclei without significant pleomorphism. The tumor cells were cytologic, similar to the cells obtained in all Neu-induced mammary tumors. Arrow, intravascular tumor emboli in the center of a dilated vessel (left). C, percentage of tumor-bearing mice with lung metastases from the indicated genotypes. Neu-YC, n = 32; and Neu-YE, n = 58.
converted to log 2 ratios. Within array normalization was done using two-
dimensional loess correction and intensity-dependent loess correction of
log ratios (18). The resulting data sets were scale normalized (19). Class
distinction was done using Linear Models for Microarray Analysis (LIMMA)
package from BioConductor (v.1.8) and R (v.2.3.0). To obtain differentially
expressed genes specific to each strain, we did class distinction between
each strain and the rest (e.g., YB versus YC, YE, YD, NPD, and NDL2-5).
Genes obtained from LIMMA were filtered for significance: B statistics 0,
Holm-adjusted P value ≤0.001, and fold change ≥2. The union of these data
sets produced a list of 1,601 unique probes representing 1,468 distinct
genemes, named “basis gene set” (Supplementary Table S1). This basis gene
set was used for further analyses. Microarray data are available online.4

### Results

**Neu-YC and Neu-YE mammary tumors exhibit morphologic similarities to Neu-YD–derived tumors but have enhanced metastatic potential.** Previously, we have investigated the capacity of different Neu autophosphorylation sites in mediating Neu-induced tumorigenesis (12). These studies revealed that transgenic mice expressing Neu-YB or Neu-YD mutant receptors exhibit significant differences in their primary mammary tumor phenotypes and lung metastasis (12). Whereas these studies indicate that these Neu-coupled signals may result in distinct tumor phenotypes, it was unclear whether mammary-specific expression of other Neu mutants would also result in discrete tumor phenotypes.

To further assess the biological significance of these Neu-coupled signaling pathways in mammary tumorigenesis and metastasis, we generated and characterized transgenic mice expressing activated Neu mutants that specifically signal through the YC or YE autophosphorylation sites under the transcriptional control of the MMTV promoter/enhancer (Fig. 1A). Multiple independent MMTV/Neu-YC and MMTV/Neu-YE transgenic lines were generated by pronuclear injection of fertilized mouse zygotes. The specificity of transgene expression was determined by analyzing total RNA from the mammary epithelium from progeny using reverse transcription-PCR (RT-PCR) with oligonucleotides directed to the SV40 component of the transgene. Of the seven founder strains derived from the MMTV/YE construct, two independent lines (YE4 and YE7) expressed the transgene in mammary epithelium. Only one of the eight MMTV/YC founder animals showed mammary epithelium-specific transgene expression due in part to an inability of several of the founders to pass the transgene to their progeny. Furthermore, we performed expression analyses on other tissues to characterize the specificity of the Neu-YC and Neu-YE transgenes. The results revealed that the main sites of transgene expression in both strains included the mammary and salivary glands, whereas low transgene levels were detected in the lung and liver (Supplementary Table S2).

To determine whether mammary-specific expression of these Neu mutants could result in the perturbation of mammary gland ductal development, mammary glands from 17-week-old virgin females were subjected to whole mount analyses. Examination of whole mounts from Neu-YC females revealed multiple histologic abnormalities, including ductal ectasia with multilayered epithelial cells and foci of microacinar structures (Fig. 1B, left). Mammary glands obtained from Neu-YE females also possessed mammary epithelial hyperplasias (Fig. 1B, right). To assess if these mammary epithelial hyperplasias would progress into neoplasias, cohorts of virgin females from each strain were monitored for mammary tumor development. Both mouse models rapidly developed multifocal mammary tumors with 100% penetrance (Fig. 2A). The Neu-YC strain developed palpable tumors with an average latency of 133 days, whereas the two independent Neu-YE lines developed tumors with latency of 126 (YE7) and 119 (YE4) days, respectively (Fig. 2A). Histologic analyses of mammary tumors from both transgenic mouse models revealed that they exhibited a solid nodular tumor pattern identical to that observed with the parental NDL2-5 strain (Fig. 2B; ref. 8). We further determined whether the Neu-YC and Neu-YE tumors possessed similar rates of lung metastasis. The lungs from tumor-bearing animals were subjected to histologic analyses for the presence of metastatic lesions. To ensure that any differences in metastatic load were not due to differences in tumor burden, all tumor-bearing animals were sacrificed, and their lungs were

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Table 1. Comparison of mammary tumor onset, metastatic potential, penetrance, and morphology in MMTV transgenic virgin females expressing various Neu phosphorylation site mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Average age of tumor formation (d)*</th>
<th>Penetrance of tumors †</th>
<th>Mammary tumor morphology</th>
<th>% of mice with lung lesions †</th>
<th>Average number of lung metastasis per lobe ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>YC</td>
<td>135 ± 14 (43)</td>
<td>100 (43)</td>
<td>Multifocal, solid nodular</td>
<td>34 (11/32)</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>YE</td>
<td>124 ± 15 (60)</td>
<td>100 (60)</td>
<td>Multifocal, solid nodular</td>
<td>40 (23/58)</td>
<td>2.6 ± 0.95</td>
</tr>
<tr>
<td>YB</td>
<td>194 ± 55 (25)</td>
<td>100 (25)</td>
<td>Focal, papillary</td>
<td>50 (7/14)</td>
<td>16.6 ± 5.3</td>
</tr>
<tr>
<td>YD</td>
<td>121 ± 20 (41)</td>
<td>100 (41)</td>
<td>Multifocal, solid nodular</td>
<td>15 (3/20)</td>
<td>0.74 ± 0.44</td>
</tr>
<tr>
<td>NYPD</td>
<td>257 ± 70 (15)</td>
<td>60 (9/15)</td>
<td>Focal, solid nodular</td>
<td>43 (6/14)</td>
<td>2 ± 0.77</td>
</tr>
<tr>
<td>NDL2-5</td>
<td>199 ± 21 (24)</td>
<td>100 (24)</td>
<td>Multifocal, solid nodular</td>
<td>50 (5/10)</td>
<td>2.5 ± 0.71</td>
</tr>
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*Average age (d) of palpable mammary tumors ± SD; parentheses indicate the numbers of animals with mammary tumors.
† Penetrance of mammary tumors in percent, determined in virgin female mice; parentheses indicate the number of animals that developed mammary tumors within 365 d over the number of observed animals.
‡ Percentage of virgin females possessing lung lesions that were tumor bearing for 5 to 7 wks; parentheses indicate the numbers of animals with lung lesions over the number of examined animals.
‡Average number of lung metastasis per lobe ± SE, eight step sections 50 µm of the lung were analyzed.
harvested at 6 weeks after the initial detection of palpable tumors. Overall, both mouse models displayed a similar metastatic potential with 34% of Neu-YC–expressing females and 40% of Neu-YE mice exhibiting lung metastatic lesions (Fig. 2C). Thus, like the parental NDL2-5 strain, mammary-specific expression of either the Neu-YC or Neu-YE transgene results in the efficient induction of metastatic mammary tumors.

We have previously shown that mammary epithelial expression of various activated Neu mutants (NDL, YB, YD, and NYPD; Fig. 1A) are capable of inducing mammary tumors (8, 12). However, mice expressing Neu-YB and Neu-YD displayed striking differences in tumor outgrowth, morphology, and metastasis relative to the parental NDL2-5 strain. These observations argued that the recruitment of specific adaptors, such as Grb2 (YB) and Shc (YD), to the receptor regulates distinct biological processes controlling Neu-induced mammary tumorigenesis and metastasis.

Like the Neu-YD strain, Neu-YC and Neu-YE transgenic mice exhibited similar tumor phenotypes (Table 1). All three transgenic strains developed mammary tumors with a shorter latency compared with NDL2-5, Neu-YB, and Neu-NYPD females. Moreover, Neu-YC–, Neu-YD–, and Neu-YE–induced tumors displayed a solid nodular morphology similar to that observed in the parental NDL2-5 strain. However, unlike the Neu-YD strain, which developed significantly fewer metastases, the Neu-YC– and Neu-YE–induced mammary tumors displayed a similar metastatic potential compared with the parental strain (Table 1; Supplementary Fig. S1A). By contrast to the nodular tumor morphology exhibited by these strains, the Neu-YB tumors displayed a distinct papillary morphology that was associated with a high rate of metastatic lesions (Table 1; ref. 12). Given that Neu-YB tumors were focal in nature, the numbers of lung metastases (~17 metastases per lobe) obtained in the Neu-YB mice were significantly higher in comparison to the other Neu mouse models (~1–3 metastases per lobe; Table 1; Supplementary Fig. S1A).

To ensure that these pathologic differences are not due to variable transgene expression, the relative Neu transcript level before the tumor onset was analyzed in mammary glands of 6-week-old females by quantitative RT-PCR. The low transgene expression levels were mainly due to low epithelial cell content at this stage of mammary gland development, but they are comparable within the different mutant strains (Supplementary Fig. S1B). Taken together, these data indicate that whereas there are several important similarities between these mammary tumor types, the Neu-YB and Neu-YD–induced tumors have distinct metastatic phenotypes.

Neu-YC– and Neu-YE–induced mammary tumors activate MAPK and phosphoinositide-3-kinase signaling pathways. To ensure that YC- and YE-derived mammary tumors expressed activated Neu, Neu immunoprecipitates from these tumor lysates were subjected to immunoblot analyses using Neu-specific and phosphotyrosine antibodies. Neu-YC and Neu-YE tumors showed Neu overexpression and tyrosine phosphorylation to levels comparable to NDL2-5, Neu-YB, and Neu-YD tumors (Fig. 3A, top). Conversely, Neu-NYPD tumors displayed lower levels of tyrosine phosphorylation, reflecting the removal of the five major in vivo tyrosine-autophosphorylation sites from the cytoplasmic tail (12).

We have previously shown that Neu mutants are capable of activating known ErbB-2 downstream signaling pathways such as the MAPK and the phosphoinositide-3-kinase (PI-3 kinase) pathways in vitro and in vivo (11, 12, 20). To assess whether Neu-YC– and Neu-YE–derived mammary tumors activate the MAPK pathway, tumor lysates were subjected to immunoblot analyses using MAPK

Figure 3. Activation of Neu downstream signaling pathways in Neu-YC– and Neu-YE–derived mammary tumors. A, Neu was immunoprecipitated (IP) from indicated mammary tumor lysates, electrophoresed, and subjected to Neu and phosphotyrosine (pTyr) immunoblot (IB) analyses (A, top). About 50 μg of the same lysates were subjected to MAPK, phospho-MAPK, and Grb2 immunoblot analyses (A, bottom). B, ErbB-3 was immunoprecipitated from indicated mammary tumors and subjected to ErbB-3 and phospho–ErbB-3 immunoblot analyses (B, top). The same protein lysates (50 μg) were analyzed for levels of Neu, phospho-Neu (Tyr877), and tubulin by immunoblot analysis (B, bottom).
and phospho-MAPK–specific antibodies. Neu-YC and Neu-YE tumors expressed comparable levels of activated MAPK to those derived from NDL2-5, Neu-YB and Neu-YD females (Fig. 3A, bottom). By contrast, Neu-NYPD tumors showed considerably less MAPK expression and phosphorylation correlating with the reduced tyrosine phosphorylation of Neu (Fig. 3A, bottom). In addition, we obtained similar levels of Akt expression and activation in all Neu-induced tumors (data not shown). Taken together, these findings suggest that Neu-YC and Neu-YE mutants can efficiently activate proliferative and cell survival signaling pathways.

Previous studies have shown that many ErbB-2 overexpressing tumors exhibit elevated levels of tyrosine-phosphorylated ErbB-3 (8, 12). To determine whether Neu-YC and -YE–expressing tumors display elevated ErbB-3 and tyrosine phosphorylation levels, ErbB-3 immunoprecipitates were subjected to immunoblot analyses using antibodies specific to ErbB-3 and phosphotyrosine. All Neu-YC and Neu-YE tumors overexpressed ErbB-3 with elevated tyrosine phosphorylation at comparable levels to that observed in Neu-YB, Neu-YD, and parental NDL2-5 tumors (Fig. 3B, top). Conversely, Neu-NYPD mammary tumors similarly overexpressed ErbB-3 but exhibited significantly lower levels of tyrosine-phosphorylated ErbB-3.

One possible explanation for the reduced ErbB-3 tyrosine phosphorylation in the Neu-NYPD tumors is that the mutation of the five major tyrosine sites in this mutant results in impaired Neu catalytic activity. The Tyr877 residue, which is located within the kinase domain, correlates with ErbB-2 kinase activity (21). Therefore, we measured the phosphorylation status of Tyr 877 in the various tumor lysates using a phosphospecific antibody to Tyr877. By contrast to the robust Tyr877 phosphorylation observed in the parental, Neu-YB, Neu-YC, Neu-YD, and Neu-YE mice, the majority of Neu-NYPD tumors displayed significantly lower levels of phosphorylated tyrosine 877 (Fig. 3B, bottom). Taken together, these observations suggest that the elimination of the five autophosphorylation sites (YA-YE) may affect the intrinsic tyrosine kinase activity of the receptor.

Neu mutant-induced tumor phenotypes display distinct transcriptional profiles. Whereas our biochemical analyses suggest the different Neu mutant-coupled adaptors can robustly activate core MAPK and PI-3’kinase signaling, the individual Neu mutant-induced tumors exhibit distinct pathologic features (Table 1; ref. 12). To further elucidate the molecular basis for these distinct phenotypes, we subjected RNA derived from all Neu mutant mammary tumors to global transcriptional profiling using mouse oligonucleotide microarrays. Using one-dimensional hierarchical cluster analyses, we classified the tumor transcriptional profiles. Including Neu-YB and Neu-YD, and parental NDL2-5 strain and group II, a majority of the Neu-YB mammary tumors exhibited increased transcriptional changes (Fig. 4A). These analyses revealed three main clusters with genes showing increased transcriptional abundance significant for Neu-YB (cluster A) and Neu-NYPD (clusters B and C). We identified ~300 Neu-YB genes that were induced 2-fold or more (Fig. 4B, cluster A). This list includes genes encoding chemokine ligands (Cxc12, Cxcl5, Cxcl10, Cc4, Ccl5, Ccl7, and Ccl20), members of the metalloproteinase family [matrix metalloproteinase 3 (MMP3), MMP11, MMP13, MMP14, Adamts2, Adams5, Adam8, Adams16, and Adam23], and transcription factors such as members of the Snail family, Snail1 and Snail2, and Twist. Interestingly, all significant differentially expressed chemokines in the Neu mutant-derived expression profiles displayed elevated transcript levels only in the Neu-YB tumors (Fig. 4C), whereas more than 50% (8:13) and 38% (5:13) of the metalloproteinases were up-regulated in Neu-YB and Neu-NYPD tumors, respectively (Fig. 4C). The majority of metalloproteinases showed no significant expression in the Neu-YC mutant, whereas decreased transcript levels were obtained in Neu-YD and Neu-YE tumors (Fig. 4C). Given that these genes have been linked to tumor progression and invasiveness (22–26), the data argue that the Neu-YB–derived tumors have acquired a transcriptional profile that is consistent with their invasive phenotype.

Among Neu-NYPD–induced genes (Fig. 4B, cluster B) was a group of genes (~7%) encoding keratins and keratin-associated proteins (Krt1-1, Krt1-3, Krt1-4, Krt1-15, Krt1-29, Krt2-6g, Krt2-19, Krtap2-4, Krtap9-1, Krtap9-2, Krtap3-2, Krtap3-3, Krtap4-7, Krtap5-1, Krtap6-1, Krtap8-1, Krtap14, Krtap16-7, Krtap16-10, Krtap16-9, and stratifin; 14:3-3c; Fig. 4B, cluster C). These proteins are involved in cellular processes such as cell cycle, motility, cell-cell contact, apoptosis, stress response, and transcriptional regulation (27, 28).

Although our microarray analyses suggest that Neu-YB–derived tumors have acquired a transcriptional profile that is consistent with their invasive nature, the other Neu mutant-induced tumors have the capacity to also colonize the lung, indicating that these tumors may require a molecular metastatic program that differs from the Neu-YB model system. To evaluate whether Neu-YB or Neu-YC, Neu-YD, Neu-YE, and Neu-NYPD signatures have relevance to human lung breast cancer metastases, we compared primary breast cancer metastasis signatures to the Neu-mutant–derived transcriptional profiles (29–34). This comparison revealed minor correlation for Neu-YC, Neu-YD, and Neu-YE–derived mammary tumors, whereas few metastasis genes were significantly altered in Neu-YB and Neu-NYPD mutants (Supplementary Fig. S2).

Strikingly, when the 54-gene lung-specific metastasis signature was compared with the Neu mutants, a substantial overlap was observed in the Neu-YB mouse model. This overlap included prognostic genes such as latent transforming growth factor-β binding protein, Ltbp1, and the angiopoietin-like protein, Angpt8, and genes mediating lung metastasis like the vascular cell adhesion molecule, VCAM1, and the inhibitor of DNA binding 1, Id1 (Fig. 4D; ref. 29). Taken together, these findings suggest that the Neu-YB tumors share a common transcriptional profile with metastatic human breast cancers that seed the lung.

Neu-YB–induced mammary tumors express elevated levels of CXCL12/SDF-1α. Another potential explanation for the enhanced metastatic behavior of the Neu-YB tumors is the striking increase in a number of chemokines that have been implicated in cell migration (Fig. 4B and C, cluster A). In particular, the up-regulation of the chemokine CXCL12/SDF-1α was noted. CXCL12/SDF-1α has been previously shown to be an important positive modulator of metastatic progression (22, 35). To confirm that CXCL12/SDF-1α was preferentially up-regulated in Neu-YB–mediated tumors, we examined CXCL12/SDF-1α levels using immunohistochemical and quantitative ELISA approaches with CXCL12/SDF-1α–specific antibodies. Immunohistochemical analyses revealed that the majority of the Neu-YB mammary tumors exhibited increased CXCL12/SDF-1α expression in epithelial cells (Fig. 5, Supplementary Fig. S3). The other Neu mutants expressed CXCL12/SDF-1α mainly in stromal cells, and only few epithelial cells were detected positive for the ligand (Fig. 5). Furthermore, we measured intracellular CXCL12/SDF-1α levels in Neu mutant–derived tumors.
using quantitative ELISA assays. Consistent with the immunohistochemical analyses, the majority of Neu-YB tumors expressed elevated levels of CXCL12/SDF-1α (Supplementary Fig. S4A). The signaling receptor for CXCL12/SDF-1α is CXCR4, which is widely expressed on lymphoid and expressed at elevated levels in breast cancer cells (35). Consistent with these previous studies, we confirmed the expression of CXCR4 in all Neu-mutant mammary tumors by immunohistochemistry and immunoblotting analyses (Supplementary Fig. S5). In addition to CXCL12/SDF-1α/CXCR4 axis, we showed that the majority of Neu-YB tumors also expressed the CCL20/macrophage inflammatory protein 3α (MIP-3α; Supplementary Fig. S4B). Given that CCL20/MIP-3α has been implicated in pancreatic and colorectal cancer metastasis (36, 37), it is conceivable that the elevated levels of this chemokine may also be involved in the Neu-YB metastatic phenotype. Collectively, the abundant expression of these molecules suggests that they may be part of the molecular metastatic program in Neu-YB–induced tumorigenesis.

Figure 4. Global transcriptional response of activated Neu mutants. A, unsupervised hierarchical clustering of the “basis gene set” consisting of 1,468 distinct genes (see Materials and Methods for details). Dendrogram, similarities between individual Neu mutant mammary tumors based on their transcriptional response. B, heat map of the “basis gene set”. Similarities between gene expression patterns are represented by the horizontal dendrogram; the vertical dendrogram represents similarities between the different Neu mutants. The relative gene expression is represented with respect to the parental NDL2-5 strain. Expression levels are color coded: red, expression above; green, expression below the mean of normalized gene expression across all samples (row Z score). a, YB-specific genes (308 genes); b, NYPD-specific changes (284 genes); c, contains genes (143) that are differentially expressed in NYPD and YE. C, heat map of the total number of chemokines and metalloproteinases with significantly differential expression in one of the Neu mutant-derived mammary tumors. The numbers of genes with significant increase in transcript levels over the total number of genes are indicated below the heat map. D, comparison of the lung-specific metastasis signature to the Neu mutant transcriptomic profile. The 54-gene human metastasis signature (29) corresponds to 39 mouse homologues. The probes are ordered by the direction of differential expression of the metastasis gene signature indicated on the left side of the heat map; red, up-regulated genes; green, down-regulated genes. The relative expression level of these genes in the various Neu mutants is represented with respect to the parental strain and color coded according to the row Z score.
Discussion

Engagement of individual Neu-coupled signals results in common and distinct pathologic features and transcriptional signatures. ErbB-2–mediated cellular proliferation and differentiation depends on its capacity to associate with various adaptor proteins, resulting in the activation of downstream transforming signals. Four independent autophosphorylation sites within the cytoplasmic tail of the receptor are capable of transducing such signals through binding of distinct adaptors (11). Two of these sites, YB and YD, are capable of efficiently inducing mammary tumors that differ in morphology and metastatic potential (12). These observations suggested that the recruitment of specific adaptors, Grb2 or Shc, have dramatic biological effects in Neu-mediated mammary tumorigenesis and metastasis. To further explore the biological relevance of the remaining two tyrosine-autophosphorylation sites, YC and YE, in Neu-induced tumorigenesis and metastasis, we have generated transgenic mice that specifically signal through either one of these sites. Both transgenic mouse strains efficiently developed multifocal mammary tumors that exhibit similar tumor phenotypes and metastatic potential (Fig. 2). Moreover, a detailed comparison of pathologic and transcriptional profiles among all Neu mutant mouse models revealed that signaling through distinct adaptor proteins results in similarities but also dramatic differences in mammary tumorigenesis, lung metastasis, and gene expression (Table 1, Fig. 4A and B). Interestingly, Neu-YC, Neu-YD, and Neu-YE strains developed mammary tumors with a shorter latency period than the parental NDL2-5 strain. Given that NDL2-5 strain still carries the negative regulatory tyrosine residue 1028 (site A; Fig. 1A; refs. 11, 38), which is missing in these Neu mutants, the shorter latency period is likely due to the loss of this key negative regulatory tyrosine.

Figure 5. Abundant expression of chemokine ligand 12 (CXCL12/SDF-1α) in epithelial Neu-YB–derived mammary tumors. Immunohistologic staining of NDL2-5, Neu-YB, Neu-YC, Neu-YD, Neu-YE, and Neu-NYPD–derived mammary tumors with CXCL12 antibody. A higher number of CXCL12-positive epithelial cells are seen in Neu-YB tumors (open arrows). CXCL12-positive stromal cells are obtained in all indicated tumors (closed arrows).
By contrast to the parental NDL2-5 strain, Neu-YB and Neu-NYPD mammary tumors exhibited distinct pathologic profiles compared with the other Neu mutants, including low tumor burden, long latency period, papillary morphology (Neu-YB), and a high incidence of lung metastasis (Neu-YB; Table 1; ref. 12). Transcriptional analyses revealed strain-specific changes in gene expression, including genes important for tumor invasion (Neu-YB; Fig. 4). Consistent with the metastatic nature of Neu-YB tumors, we found that the YB transcriptomic signature exhibits a striking similarity with previously published lung-specific human breast cancer metastasis signatures (Fig. 4D). This suggests that Neu-YB signaling may act through a common pathway used in human breast cancers. However, given that the other Neu mutants also develop lung metastases, there are likely alternative signaling pathways resulting in the metastatic phenotype. In this regard, it is interesting to note that there are numerous nonoverlapping metastatic signatures that have been proposed for metastatic breast cancer.

Multiple redundant tyrosine-autophosphorylation sites in Neu activate MAPK and PI-3 kinase signaling pathways. Biochemical analyses of all Neu mutant-induced mammary tumors revealed the activation of the MAPK and the PI-3′kinase pathway (Fig. 3A: refs. 8, 12). Like the parental NDL2-5 tumors, all Neu mutant tumors expressed elevated levels of ErbB-3 (Fig. 3B). However, unlike the other Neu mutants, NYPD tumors exhibited significantly reduced tyrosine phosphorylation (Fig. 3B). This low basal level of ErbB-3 transphosphorylation was further correlated with impaired phosphorylation of a key tyrosine residue Y877 located within the activation loop of the catalytic domain of Neu. Indeed, a recent study showed that the tyrosine residue Y877 of the ErbB-3 receptor may play an important role in the regulation of ErbB-2 signaling (21). These observations suggest that the Neu-NYPD mutant possesses reduced kinase activity resulting in low phosphorylation of the kinase dead ErbB-3 receptor.

Consistent with these findings, recent studies have suggested that the COOH-terminal phosphorylation domain of ErbB-1 negatively influences receptor kinase activity (39, 40). A direct contact between the kinase and a segment from the cytoplasmic domains has been proposed, which inhibits the enzymatic activity (39, 40). Moreover, tyrosine phosphorylation results in increased mobility of the cytoplasmic domain and its displacement from the kinase core (41, 42). Together with our findings, these data suggest that it is likely that the elimination of all major tyrosine phosphorylation sites in Neu-NYPD leads to conformational changes, resulting in a decreased kinase activity. On the other hand, only one phosphorylated tyrosine may be sufficient enough to displace the cytoplasmic tail from the kinase domain and thereby activate the kinase.

Neu-YB-specific expression of genes involved in migration and invasiveness. Pathologic and transcriptional studies suggest the Neu-YB transgenic mouse model displays a more aggressive phenotype among all Neu tyrosine phosphorylation mutants (Table 1, Fig. 4; ref. 12). This argument is supported by the identification of genes important for metastasis that were significantly induced in Neu-YB tumors, including metalloproteinases and the transcription factors Twist and Snail (Fig. 4B, cluster A). Given that these genes have been linked to tumor progression and invasiveness of breast cancer cells (43–46), it is conceivable that they may play a comparable role in Neu-YB metastasis. Indeed, it has been shown that Snail regulates the expression of metalloproteinases (47–49).

Chemokines are a second set of YB-specific response genes (Fig. 4B and C, cluster A). This finding was of particular interest due to the previously shown role of chemokines and their receptors in cancer cell migration, invasion, and organ-specific metastasis (22, 50–53). Particularly, CXCL12/SDF-1a and its receptor CXCR4 have been recently implicated in breast cancer metastasis (22, 51). Similarly, elevated levels of CXCL12/SDF-1a have also been detected in ovarian tumor epithelial cells (54). Consistent with these studies, we have detected abundant expression of CXCL12/SDF-1a in mammary epithelial cells of Neu-YB tumors compared with the other Neu-mutant–derived mammary tumors (Fig. 5, Supplementary Fig. S4A). In addition to CXCL12/SDF-1a, we have also detected abundant CCL20/MIP-3a levels in the Neu-YB tumors (Supplementary Fig. S4B). The elevated levels of the key chemokines in the Neu-YB tumors may facilitate metastatic progression through a number of distinct mechanisms. One potential mechanism involves chemokines mediating an increased secretion of metalloproteinases that are involved in the degradation of extracellular matrix molecules and thereby promoting invasion and metastasis (55, 56). Consistent with this model is the abundant expression of various members of the metalloproteinase family in the Neu-YB tumors (Fig. 4B and C, cluster A).

Alternatively, chemokines such as CXCL12/SDF-1a and CCL20/MIP-3a are known to be potent regulators of inflammatory immune cell function. In this regard, it is evident that inflammatory cells such as macrophages are important positive modulators of metastatic behavior of tumor cells (57). Future studies directed toward ablating these key cytokines in Neu-induced tumors should provide important insight into the relative contribution of these inflammatory molecules.

Our observations have important implications for the understanding of the molecular basis for the potent transforming properties of ErbB-2 in the mammary epithelium. Given that morbidity of breast cancer is associated with increased metastatic behavior, our data suggest that signaling pathways activated by an oncogenic receptor such as Neu are important parameters in dictating its capacity to metastasize. The observation that the Neu-YB mutant displayed a distinct metastatic phenotype is supportive of this concept. Unlike the other Neu autophosphorylation mutants, the Neu-YB mouse model exhibited a higher rate of lung metastasis, which was reflected by the Neu-YB–specific expression of important determinants of metastatic breast cancer such as CXCL12/SDF-1a, metalloproteinases, Twist, and Snail (22, 44–46). This suggests that the direct recruitment of Grb2 to the Neu receptor has distinct phenotypic and transcriptional responses compared to the other Neu-coupled signaling molecules. Interestingly, recent studies emphasize the importance of epithelial-stromal/myoepithelial interactions in breast cancer tumorigenesis with CXCL12/SDF-1a acting as a mediator (58, 59). Thus, our in vivo studies represent a more physiologic environment that incorporates these essential interactions and may account for the different tumor morphologies of the Neu mutant mouse models. Future mouse studies designed to investigate the signaling specificity of HER2-positive human breast cancers may provide important insight into the metastatic potential of these tumors.

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ErbB-2 Signals Promote Distinct Tumor Signatures


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