ORIGINAL ARTICLE

The ErbB2ΔEx16 splice variant is a major oncogenic driver in breast cancer that promotes a pro-metastatic tumor microenvironment

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Amplification and overexpression of *erbB2/neu* proto-oncogene is observed in 20–30% human breast cancer and is inversely correlated with the survival of the patient. Despite this, somatic activating mutations within *erbB2* in human breast cancers are rare. However, we have previously reported that a splice isoform of *erbB2*, containing an in-frame deletion of exon 16 (herein referred to as ErbB2ΔEx16), results in oncogenic activation of *erbB2* because of constitutive dimerization of the ErbB2 receptor. Here, we demonstrate that the ErbB2ΔEx16 is a major oncogenic driver in breast cancer that constitutively signals from the cell surface. We further show that inducible expression of the ErbB2ΔEx16 variant in mammary gland of transgenic mice results in the rapid development of metastatic multifocal mammary tumors. Genetic and biochemical characterization of the ErbB2ΔEx16-derived mammary tumors exhibit several unique features that distinguish this model from the conventional ErbB2 ones expressing the *erbB2* proto-oncogene in mammary epithelium. Unlike the wild-type ErbB2-derived tumors that express luminal keratins, ErbB2ΔEx16-derived tumors exhibit high degree of intratumoral heterogeneity co-expressing both basal and luminal keratins. Consistent with these distinct pathological features, the ErbB2ΔEx16 tumors exhibit distinct signaling and gene expression profiles that correlate with activation of number of key transcription factors implicated in breast cancer metastasis and cancer stem cell renewal.

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INTRODUCTION

Amplification and elevated expression of the *erbB2* proto-oncogene is observed in 20–30% of human breast cancers and is inversely correlated with the survival of the patient.^{1–3} Direct evidence supporting a role for ErbB2 in mammary tumorigenesis derives from studies with transgenic mice expressing the *erbB2* proto-oncogene under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer. Mammary epithelial expression of *erbB2* oncogene results in the rapid induction of multifocal mammary tumors.^{4,5} By contrast, mammary epithelial expression of the proto-oncogenic form of ErbB2 (herein referred as wild-type ErbB2) resulted in induction of focal mammary tumors after a long latency period.⁶ Tumor progression in these strains is associated with the activation of the ErbB2 tyrosine kinase activity because of the occurrence of somatic activating mutations in the transgene in the majority of the mammary tumors analyzed.^{6–9} Significantly these mutations are confined to a cysteine-rich region of the receptor located in a juxtatransmembrane domain and comprises either deletion or insertion of single cysteine residues. Further genetic and biochemical analyses have revealed that these cysteine alterations promote the formation intermolecular cysteine bridges between ErbB2 monomers, resulting in constitutive receptor dimerization and activation.⁹

Although comparable somatic mutations in *erbB2* have not been detected in human breast cancer, several studies have reported the expression of an alternatively spliced *erbB2* isoform (ErbB2ΔEx16) that carries a 16-amino-acid in-frame deletion in the juxtatransmembrane domain closely mimics the sporadic *erbB2* transgene mutations observed in the transgenic mice expressing the *erbB2* proto-oncogene.^{10,11} Like sporadic transgene mutations, this *erbB2* splice variant is constitutively active because of its capacity to form disulfide-bonded dimers.¹¹ In addition to having an important role in the oncogenic activation of ErbB2 receptor, it has been recently reported that the ErbB2ΔEx16 isoform is the principal target of the anti-ErbB2 therapeutic monoclonal Trastuzumab.^{12,13}

To further validate the biological significance of the ErbB2ΔEx16 variant in mammary epithelium, we established a transgenic mouse model that expresses the ErbB2ΔEx16 variant in an inducible manner in the mammary epithelium. In contrast to the transgenic mice expressing the wild-type ErbB2 receptor in the mammary epithelium that developed focal mammary tumors after a long latency period, the ErbB2ΔEx16 strains rapidly developed multifocal mammary tumors that frequently metastasized to the lung. Remarkably, in contrast to the ErbB2 tumors that uniformly expressed the luminal keratin 8 marker, the ErbB2ΔEx16-derived tumors exhibited a high degree intratumoral heterogeneity

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expressing both luminal and basal keratins. Consistent with the basal origin of these tumors, gene expression analyses revealed that in contrast to the luminal pattern for ErbB2, the ErbB2ΔEx16-derived tumors possessed many features of basal breast cancer. Molecular and genetic analyses of these tumors further showed that the basal molecular phenotype was due to selective signaling of the c-Src family and p38 kinases by the ErbB2ΔEx16 variant that in-turn resulted in activation of the Smad2, HIF1α, Stat3 and YB-1 transcriptional networks. Taken together, these observations suggest that the ErbB2ΔEx16 isoform possesses distinct signaling and cellular localization processes that may impact on its role in mammary tumor progression.

RESULTS

The ErbB2ΔEx16 isoform is expressed at different levels in a number of ErbB2-positive cell lines and confers transforming activity *in vivo*

To confirm the prevalence of the ErbB2ΔEx16 isoform in ErbB2-positive category of breast cancer, we used a RNA-seq approach to simultaneously measure the abundance of the ErbB2ΔEx16 compared with wild-type transcript ErbB2 transcript in a collection of established breast cancer cell lines representing different breast cancer subtypes¹⁴ (Figure 1a). The results revealed that the abundance of the ErbB2ΔEx16 isoform varied considerably with different ErbB2-positive cell lines. Although low levels of ErbB2 spliced form were detected in the SKBR3 (1.8% of the total ErbB2 transcript), relatively high levels of the ErbB2ΔEx16 were seen in the ZR7530 cells (12% of the total ErbB2 transcript) (Figure 1b). Other ErbB2 cell lines maintained a modest level of the spliced variant (4–5% of total ErbB2). Although the proportion of ErbB2ΔEx16 variant varied in ErbB2-positive lines, because of amplification of ErbB2, the absolute levels of the ErbB2ΔEx16 variant were high by comparison with other breast cancer cell lines representing other breast cancer subtypes (Figure 1c).

Interestingly, the expression of the ErbB2ΔEx16 form was not restricted to tumor cells. Using reverse transcriptase-PCR that allowed the simultaneous measurement of wild-type ErbB2 and ErbB2ΔEx16 transcripts, we showed that the ErbB2ΔEx16 variant could be detected at variable levels (2–10% of total ErbB2 transcript) in a variety of normal human tissues (Supplementary Figure S1). Together, these observations indicate that both wild-type ErbB2 and ErbB2ΔEx16 transcripts are expressed in both normal and malignant tumor samples and that breast cell lines with amplified ErbB2 possessed particularly elevated levels of the ErbB2ΔEx16 variant.

Although these observations suggest that the ErbB2ΔEx16 transcript is expressed at elevated levels in ErbB2 amplified breast cancer cell lines, the biological significance of these observations was unclear. To directly test the transforming properties of this variant, we established independent clones of immortalized NMuMG mammary cells that expressed either constitutively active rat ErbB2 (NeuNT),¹⁵ wild-type human ErbB2 or the human ErbB2ΔEx16 variant at comparable levels (Supplementary Figure S2A). To evaluate whether cells expressing the ErbB2 variants exhibited differential tumorigenic potential, mammary cells derived from two independent clones expressing either wild-type ErbB2, activated ErbB2 (NeuNT) or ErbB2ΔEx16 were injected orthotopically into immunodeficient recipient mice and their growth was monitored (Supplementary Figure S2B). In contrast to the rapid onset of tumors seen in both clones expressing NeuNT and the ErbB2ΔEx16, two independent clones of cells expressing the wild-type form of ErbB2 failed to form tumors (Supplementary Figure S2B). These observations argue that unlike the ErbB2ΔEx16 and activated forms of ErbB2 (NeuNT), elevated expression of wild-type ErbB2 is not sufficient to convert these immortalized cells to the malignant phenotype.

Although wild-type ErbB2 appears to be incapable of transforming immortalized epithelial cells, an outstanding question is whether this isoform is capable of maintaining the tumorigenic state. In order to test this, NMuMG cells were used to generate cell lines expressing a doxycycline-inducible ErbB2ΔEx16, isolating cells with tightly regulated induction (Figure 2b). In the presence of doxycycline, robust ErbB2ΔEx16 protein expression was observed as quickly as 24 h after *in vitro* doxycycline administration (Figure 2a). These cells were subsequently engineered to express wild-type ErbB2 as well, which were selected using fluorescence-activated cell sorting in the absence to doxycycline, in order to sort cells by robust wild-type ErbB2 expression. Cells were injected orthotopically into athymic nude mice, and the mice were treated with 2 mg/ml doxycycline in the drinking water. Consistent with previous results, ErbB2ΔEx16 induction drives rapid transformation and tumor outgrowth (Figure 2c). However, when doxycycline was withdrawn, the tumors failed to express ErbB2ΔEx16 but retained robust expression of wild-type ErbB2 (Figure 2b), leading to rapid regression of the established mammary tumors (Figure 2d). These observations demonstrate that expression of wild-type ErbB2 is insufficient to maintain a tumorigenic state in the absence of ErbB2ΔEx16 expression and indicate that the ErbB2ΔEx16 isoform is a major oncogenic driver.

To determine whether the differential transforming activity of the ErbB2ΔEx16 variant involved altered receptor recycling, we performed immunofluorescence staining on cells expressing the different ErbB2 receptors with antibodies directed toward ErbB2 and the early endosome marker EEA1 (Figure 3a). Cells were treated with cycloheximide in serum-free medium for 1 h, followed by 1 h culture in complete medium at 4 °C. To permit receptor internalization, cells were warmed to 37 °C with fresh complete medium for 15 min. Examination of NeuNT-expressing cells revealed that ErbB2 and EEA1 were colocalized in the early endosome, indicating rapid downregulation of ErbB2 from the cell surface (Figure 3a). In contrast the ErbB2ΔEx16 variant could not be detected in the endosomal compartment.

To confirm that lack of detectable ErbB2ΔEx16 receptor in the endosomal compartment was due to defect in downregulation from the cell surface, we performed a biotin labeling assay of cell surface proteins and measured the percentage of internalized ErbB2 that was protected from proteolytic degradation. Consistent with the rapid internalization of NeuNT in the endosomal compartment, a significant percentage of ErbB2 was protected 60 min after shifting the cells to the permissive temperature for sorting. By contrast, only a small proportion of the ErbB2ΔEx16 variant was internalized (Figures 3b and c).

Another indication of altered trafficking for the ErbB2ΔEx16 variant stemmed from the observation that mammary tumor cell lines derived from either transgenic mice (Figure 4) or NMuMG cells expressing the ErbB2ΔEx16 isoform were resistant to the anti-ErbB2 therapeutic T-DM1 antibody–drug conjugate, whereas control cells expressing the wild-type ErbB2 were highly sensitive to T-DM1 (Figures 3d and e). Given that T-DM1 drug delivery requires internalization, the inability of T-DM1 to kill these the ErbB2ΔEx16 expressing likely reflects its inability to be internalized.¹⁶ Collectively, these observations demonstrate that the ErbB2ΔEx16 is primarily a cell surface receptor that is not efficiently internalized and also indicate that expression of the ErbB2ΔEx16 isoform may confer resistance to T-DM1.

Mammary-specific expression of the ErbB2ΔEx16 isoform results in the rapid induction of metastatic breast cancers

To directly evaluate the oncogenic potential of the ErbB2ΔEx16 isoform in the mammary epithelium, we placed the ErbB2ΔEx16 under the transcriptional control of the tetracycline response element and crossed these strains of FVB mice to a separate FVB strain expressing the reverse tetracycline transactivator under the transcriptional control of the MMTV promoter.¹⁷ To facilitate

Figure 1. RNA Seq analysis of human breast cancer cell lines. **(a)** ErbB2ΔEx16 is an isoform of ErbB2 that lacks, by alternative splicing, the 16 amino acids encoded by Exon 16. **(b)** Consistent with previous findings, the ratio of ErbB2ΔEx16 transcript falls between 2 and 10% of total ErbB2 transcript across all cell line transcriptional subtypes. **(c)** Total levels of ErbB2ΔEx16 are enriched in ErbB2+ cell lines.

detection of transgene expressers, we placed an internal ribosome entry site preceded enhanced green fluorescent protein (IRES-EGFP) expression cassette downstream of the ErbB2ΔEx16 complementary DNA (cDNA; Figure 4a). In this manner, we can induce expression of the ErbB2ΔEx16 in the mammary epithelium by the addition of doxycycline to the drinking water. Of the initial seven independent founder lines, two independent founder strains could be induced to express elevated levels of the ErbB2ΔEx16 isoform upon doxycycline administration (Supplementary Figures S3A and S3B). Using reverse transcriptase-PCR analyses, we show that ErbB2ΔEx16 expression is primarily restricted to the mammary and salivary glands (Supplementary Figure S4).

To further characterize the kinetics of tumor onset in the inducible ErbB2ΔEx16 model, we generated cohorts of female mice from these two independent strains and monitored tumor induction following the addition of doxycycline to drinking water. Groups were unblinded throughout the study, and a target sample size of 20 per group was chosen to ensure sufficient statistical power. The results revealed that in contrast to MMTV/ErbB2 strain that developed focal mammary tumors after long latency period ($T_{50}=280$ days),¹⁸ both inducible ErbB2ΔEx16 strains rapidly grew multifocal mammary tumors with an extremely short latency period ($T_{50}=10$ and 28 days) (Figure 4b). In addition, ErbB2ΔEx16 tumors frequently gave rise

Figure 2. ErbB2 is incapable of maintaining the tumorigenic state. **(a)** Doxycycline-inducible NMuMG cells (NMuMG-iSP) rapidly express ErbB2ΔEx16 in the presence of doxycycline within 24 h of induction. **(b)** In cells expressing stable ErbB2 with inducible ErbB2ΔEx16 (NMuMG-WTiSP), quantitative PCR confirms robust expression of ErbB2ΔEx16 mRNA in the presence, but not the absence of doxycycline. By contrast, ErbB2 expression remains constant. **(c)** When NMuMG-WTiSP cells were injected orthotopically into athymic nude mice, tumors were detected 3 weeks after injection when treated with doxycycline. **(d)** Withdrawal of doxycycline results in rapid and complete regression of the WTISP tumors.

to metastatic lesions in the lung in the majority of tumor-bearing animals (Figures 4c and d).

Histological analyses revealed that the ErbB2ΔEx16-derived tumors possessed a pathology that is distinct from those expressing the *erbB2* proto-oncogene (Figures 5a and c). Using trichrome staining, which stains for collagen deposition, we confirmed that ErbB2ΔEx16-derived tumors had much higher levels of matrix deposition than the comparable ErbB2 tumors (Figures 5b and d).

To further evaluate whether the observed pathological differences between the two ErbB2 tumor models were due to different cellular origin, we next stained both full-length and the ErbB2ΔEx16-derived tumors with either luminal (cytokeratin 8) or basal keratin (cytokeratin 14, 6 and 5) markers. In contrast to tumors expressing full-length ErbB2 that were uniformly positive for keratin 8 marker and negative for any of the basal keratin markers, the ErbB2ΔEx16-derived tumors stained positive for both luminal and basal keratins (Supplementary Figure S5A). Indeed, co-staining with different keratin markers revealed a diversity of cells that co-expressed both basal and luminal keratin markers. (Supplementary Figure S5B). Collectively, these observations argue that in contrast to ErbB2-derived tumors, mammary epithelial expression of ErbB2ΔEx16 results in rapid induction of metastatic mammary tumors that possess a high degree of intratumoral heterogeneity.

To further establish the critical importance of the ErbB2ΔEx16 as major oncogenic driver in mammary tumorigenesis, we next determined whether withdrawal of doxycycline from the drinking water in ErbB2ΔEx16 tumor-bearing mice would result in tumor regression. The results showed that removal of doxycycline from tumor-bearing animals resulted in rapid regression of the ErbB2ΔEx16-derived tumors. However, after a variable tumor-free period, focal mammary tumors reappeared (Supplementary Figure S6A). Molecular and pathological analyses of these recurrent tumors revealed that exhibited epithelial-mesenchymal transition (EMT) phenotype (Supplementary Figure S6C) that was further correlated with reduced expression of the epithelial marker E-cadherin and complete loss of the ErbB2ΔEx16 transgene expression (Supplementary Figure S6B). Thus, like our previous cell culture data (Figure 2), these observations indicate that sustained expression of the ErbB2ΔEx16 is required to maintain the transformed state.

The ErbB2ΔEx16-derived tumors exhibit unique signaling and transcriptional profiles because of activation of transcription factor network

To further elucidate the molecular basis for the potent transforming ability of the ErbB2ΔEx16 isoform, we performed both reverse phase protein array (RPPA) and immunoblot analyses on a number of downstream signaling pathways. Despite the constitutive dimerization of the ErbB2ΔEx16 receptor,¹¹ the levels of tyrosine phosphorylated ErbB2, EGFR and ErbB3 were impaired when comparing to ErbB2 tumors (Figure 6a). Conversely, the ErbB2ΔEx16-derived tumors exhibited a profound activation of p38 and c-Src family kinases compared to the tumors expressing the ErbB2 receptor (Figure 6b). Another important set of phosphorylated proteins that were clearly activated in the RPPA as measured by the phosphorylation status in the ErbB2ΔEx16-derived tumors were a number of transcription factors, including Stat3, YB-1, Smad2 and HIF1α (Supplementary Figure S7). To confirm the activation status of this transcription factor network in the ErbB2ΔEx16 class of mammary tumors, we performed immunoblot analyses with phospho-specific antibodies directed against each of these transcription factors. Consistent with RPPA analyses, the results showed that although there was no absolute increase in the levels in these transcription factors, the ErbB2ΔEx16-derived tumors exhibited a marked elevation in activation status of these key transcription factors (Figure 6c).

The observations outlined above indicate that the ErbB2ΔEx16-derived tumors have pathological and molecular features that are distinct from tumors induced by the wild-type ErbB2 receptor. To further explore the molecular basis for the striking differences between these closely related ErbB2 receptors, we performed gene expression profiling on eight independent tumors from either the wild-type ErbB2 or the ErbB2ΔEx16 strains using the Agilent platform. Consistent with the molecular and pathological analyses, unsupervised hierarchical clustering of gene expression profiles revealed that the ErbB2Δ16 tumors co-clustered and could be readily distinguished from their ErbB2 counterparts (Figure 6d). Given that our RPPA data indicated that ErbB2ΔEx16 selectively activated Stat3, Smad2 and HIF1α transcription factors, we next evaluated whether differential gene expression profiles between two ErbB2 tumor subtypes could be due to selective activation of this transcriptional factor network. To accomplish this, we investigated whether known target genes of these transcription

Figure 3. ErbB2ΔEx16 displays altered internalization, which affects response to therapy. **(a)** Serum-induced internalization of NeuNT results in colocalization with EEA1, which is absent in ErbB2ΔEx16 cells. **(b)** Quantification of a biotin internalization assay **(c)** reveals that while NeuNT is effectively internalized, ErbB2ΔEx16 remains at the cell surface. **(d)** Treatment of NMuMG-ErbB2ΔEx16 cells with the anti-ErbB2 therapy T-DM1 reveals an innate resistance to treatment compared with SKBR3 cells, which express very high levels of full-length ErbB2. In addition, **(e)** NMuMG cells expressing full-length ErbB2 are intrinsically more sensitive to T-DM1 treatment than NMuMG-ErbB2ΔEx16 cells.

factors were selectively upregulated in the ErbB2ΔEx16-derived tumors. The results of these analyses confirmed that differential gene expression profile exhibited by the ErbB2ΔEx16-derived tumors can in part be accounted for the activation of this transcriptional network (Figure 7a). Target genes downstream of Smad2 ($P=0.0019$), HIF1α ($P=0.014$) and Stat3 ($P=0.008$) were significantly enriched in ErbB2ΔEx16 samples (Figures 7b–d). Notably, expression of Stat3 target genes such as interferon γ and Cxcl10 that are implicated in regulation of tumor immune microenvironment were markedly upregulated in the ErbB2ΔEx16-derived tumors. Similarly, selective upregulation of Smad2 targets such as transforming growth factor-β superfamily was also observed in the ErbB2ΔEx16-derived tumor setting (Figure 7a). In addition, transcription factors Snail1 and Twist1 that are also targets of Smad2 and HIF1α in ErbB2ΔEx16 tumors. Interestingly, these latter transcription factors are thought to have a critical role in the epithelial-mesenchymal transition process.¹⁹ Collectively, these

observations indicate that the ErbB2ΔEx16-derived tumors possess transcriptional signature associated with invasive breast cancers.

DISCUSSION

In this study, we demonstrate that the oncogenic ErbB2ΔEx16 variant is expressed at appreciable levels in a number of ErbB2-expressing breast cancer cell lines. In addition, we show that the ErbB2ΔEx16 expression is both sufficient and necessary for the maintenance of the transformed phenotype (Figure 2 and Supplementary Figure S6). Specifically, we demonstrate that in contrast to another oncogenic ErbB2 mutant (NeuNT), the ErbB2ΔEx16 isoform is constitutively localized to the plasma membrane compartment (Figure 3). Consistent with the unique properties of the ErbB2ΔEx16 variant, mammary-specific expression of ErbB2ΔEx16 results in a rapid induction of multifocal metastatic mammary tumors that possessed

Figure 4. A transgenic mouse model of ErbB2ΔEx16-driven tumorigenesis. **(a)** A reverse tetracycline-dependent transactivator (rtTA) is expressed in the mammary epithelium of transgenic mice via the MMTV promoter. In the presence of doxycycline, ErbB2ΔEx16 is expressed in mammary epithelial cells through rtTA binding to the tetracycline-dependent promoter (TetO). **(b)** Expression of ErbB2ΔEx16 drives rapid tumor onset with a latency of 28 and 10 days for two independent founder lines, compared with 280 days for full-length ErbB2. **(c)** ErbB2 and ErbB2ΔEx16 models metastasize to the lungs with a frequency of 74% and 95%, respectively, with **(d)** a significantly increased number of lung lesions in ErbB2ΔEx16 mice ($P=0.03$).

molecular and pathological features that are distinct from the conventional MMTV-ErbB2 models. These features include high levels of intratumoral heterogeneity that is characterized by co-expression of both luminal and basal keratins and is reflected at the molecular level by a distinct gene expression profile (Figures 4–7).

Although a number of studies have detected the presence of the ErbB2ΔEx16 variant in both established breast cancer cell lines and primary breast cancers (Figure 1),^{10–13} the precise role of this variant in ErbB2-induced tumor progression was unclear. Using NMuMG immortalized mammary epithelial cell model, we demonstrate that elevated expression of wild-type ErbB2 is not sufficient to drive morphological transformation of this immortalized mammary epithelial cell line (Supplementary Figure S2). By contrast, expression of the activated ErbB2 (NT mutation) or the ErbB2ΔEx16 form is sufficient to drive the tumorigenic conversion of NMuMG cells (Supplementary Figure S2). Furthermore, expression of wild-type ErbB2 in the context of an ErbB2ΔEx16-driven tumor is incapable of maintaining transformation following the loss of ErbB2ΔEx16 expression (Figure 2). These observations indicate that at least in this immortalized cell system, the ErbB2ΔEx16 is the major oncogenic driver.

The potent transforming property of the ErbB2ΔEx16 isoform are further correlated with its constitutive localization to the plasma membrane. By contrast, other activated versions of ErbB2

(NeuNT) are rapidly downregulated from the cell surface (Figures 3a and b). The difference in receptor trafficking in ErbB2ΔEx16 tumors may account for the distinct signaling output from the ErbB2ΔEx16 receptor. In this regard, it has been reported that the ErbB2ΔEx16 variant is specifically associated within a membrane compartment harboring c-Src.²⁰ Another important consequence of the differential trafficking properties of the ErbB2ΔEx16 variant, is that tumor cells expressing this variant are highly resistant to T-DM1 (Figures 3c and d). As the biological activity of Trastuzumab-drug conjugate is dependent on its ability to be internalized to release the drug toxin,¹⁶ the inability of T-DM1 to kill the ErbB2ΔEx16 variant expressing tumor cells reflects its inability to be efficiently internalized. These data contrast with a recent report indicating that Trastuzumab can effectively eliminate ErbB2ΔEx16-expressing tumors *in vivo*.¹³ The difference between the *in vitro* resistance phenotype we observed (Figure 3) and the *in vivo* sensitivity may reflect the importance of the antibody directed cellular cytotoxicity in T-DM1 antitumor response.²¹

Consistent with the unique signaling properties of the ErbB2ΔEx16 variant, mammary epithelial expression of this isoform resulted in rapid induction of multifocal mammary tumors that frequently metastasize to the lung, whereas comparable strains expressing ErbB2 in the mammary gland develop focal mammary tumors only

Figure 5. Histopathology of ErbB2-driven tumor phenotypes described by hematoxylin and eosin-stained slides (**a**, **c**) and comparing the relative amounts of extracellular matrix (ECM) deposition using green trichrome staining for connective tissue (**b**, **d**). Figures 1a and b illustrate a signature solid nodular tumor from an ErbB2 bearing mouse. In contrast, **c** and **d** illustrates the more glandular phenotype induced by the ErbB2ΔEx16 protein, which has abundant connective tissue separating the nests and cords of tumor cells. These tumors infiltrate through the adipose tissue and do not have pushing margins seen in the signature phenotype (**a**, **b**). The scale bar indicates 250 μm (**a**).

after a long onset period (Figure 4). In agreement with these observations, a transgenic line expressing the ErbB2ΔEx16 variant with constitutive MMTV promoter rapidly developed mammary tumors.²² However, detailed molecular and pathological analyses of mammary tumors were not reported.

Another distinct feature of these the ErbB2ΔEx16-expressing tumors is that they have several unique pathological features, including major deposition of extracellular matrix (Figure 5) and co-expression of both luminal and basal keratin markers (Supplementary Figure S5). The dense extracellular matrix depositions observed in the ErbB2ΔEx16-derived tumors is further correlated with the metastatic dissemination to the lung (Figures 5c and d). In many respects, this extracellular matrix deposition phenotype resembles high breast density that is a known risk factor for development of breast cancer.²³ Other studies have linked matrix density to directly impact on metastatic fate of tumor cells.^{24,25}

Although both of the ErbB2-driven mouse models express the luminal marker cytokeratin 8 (Krt8; Supplementary Figure S5), only the ErbB2ΔEx16-derived tumors exhibit expression of basal cytokeratin markers such as Krt5, Krt6 and Krt14 (Supplementary Figure S5). These findings indicate that the ErbB2ΔEx16-derived mammary tumors exhibit a high degree of intratumoral heterogeneity comprising mixed cell lineages, whereas MMTV wild-type ErbB2 tumors comprise a uniform luminal cell type.

The ErbB2ΔEx16-derived tumors also exhibit a profound difference in signaling pathways activated compared with their full-length counterparts. Using RPPA and immunoblot analyses

(Figure 6, Supplementary Figure S7), we demonstrate that the ErbB2ΔEx16-derived tumors exhibit reduced tyrosine phosphorylation of ErbB2 and its heterodimer partners EGFR and ErbB3 (Figure 6a) and elevated activation c-Src family of tyrosine kinases and activation of p38 kinase (Figure 6b) compared with ErbB2-derived tumors. These analyses also reveal that ErbB2ΔEx16-dependent signaling is responsible for activation of a number of key transcription factors including Stat3, Smad2, YB-1 and HIF1α (Figure 6c). Activation of these transcription factor networks has been implicated in regulating the epithelial-mesenchymal transition process that is involved in breast cancer stem cell renewal.^{26,27} Consistent with these biochemical analyses, analyses of the gene expression profiles of ErbB2ΔEx16-derived tumors reveal that many of the known target genes of this transcriptional network are selectively upregulated in ErbB2ΔEx16-derived tumors (Figure 7). Critically, comparison of the ErbB2ΔEx16 transcriptional signature with the human data sets reveal that they align with basal category of human breast cancers that have particularly bad outcome (Supplementary Figure S9).

One important set of Stat3 target genes that are upregulated in the ErbB2ΔEx16 tumors, such as interferon γ and Cxcl10, are important modulators of immune microenvironment (Figure 7). Given that activation of Stat3 is primarily in the tumor epithelium, these Stat3-dependent cytokines are acting in a paracrine manner to promote immune infiltration (Supplementary Figure S8). In this regard, it is noteworthy that mammary tumor-specific disruption of Stat3 in an ErbB2 model results in a decrease in infiltrating macrophages because of downregulation of a number of Stat3-

Figure 6. Immunoblot analysis of ErbB2-expressing tumors. **(a)** Full-length ErbB2-driven tumors co-express heterodimer partners ErbB3 and EGFR, whereas ErbB2ΔEx16 tumors arise in the absence of heterodimerization partners. **(b)** ErbB2ΔEx16 displays heightened activation of Src family kinases (SFK) and p38 MAPK. **(c)** In addition, ErbB2ΔEx16-expressing tumors preferentially activate a distinct subset of transcription factors, primarily Smad2, HIF1α, Stat3 and YB-1. **(d)** Unsupervised hierarchical clustering of tumors results in grouping of tumors by genotype.

dependent inflammatory cytokines.²⁸ Interestingly, the loss of an inflammatory tumor microenvironment is associated with a marked decrease in ErbB2-dependent metastasis.²⁸ Indeed, the increase in spontaneous metastasis observed in ErbB2ΔEx16-derived tumors (Figure 4) may be due to enhanced inflammatory tumor immune microenvironment. In addition to modulating macrophage infiltration, our recent studies have indicated that epithelial activation of Stat3 has a critical role in maintaining an immune-suppressive tumor microenvironment. For example, mammary epithelial deletion of Stat3 in PyV mT model results in rapid immune-mediated clearance of emerging PyV mT tumors.²⁹ Collectively, these observations argue that the potent transforming properties of ErbB2Δ16 variant is due to its impact on the tumor immune microenvironment.

Recent studies have suggested that the ErbB2ΔEx16 variant may have an important role in biological effects of ErbB2 targeted therapies, such as the anti-ErbB2 antibody Trastuzumab. In one report, mammary cell lines expressing ErbB2ΔEx16 are resistant to Trastuzumab treatment,²⁰ whereas several recent publications assert that Trastuzumab treatment can eliminate ErbB2ΔEx16-expressing tumors. Indeed, the authors of the latter set of studies have argued that the ErbB2ΔEx16 variant is the primary target of Trastuzumab.^{12,13} In light of our observations that the ErbB2ΔEx16 variant confers resistance to Trastuzumab-based T-DM1 raise the possibility that its expression may be an important determinant of its resistance to these agents. Another interesting finding from our studies is that the ErbB2ΔEx16 variant is also expressed to some level in a number of normal tissue sites (Supplementary Figure S1) indicating a potential role in normal tissue homeostasis. In this regard, we have shown that mice expressing ErbB2 cDNA (hence lacking the ErbB2ΔEx16 variant) develop both spindle and cardiac

cell dysfunction indicating that the ErbB2ΔEx16 variant may have important physiological functions.^{30,31} More recently, several RNA splicing factors have been identified to facilitate the Exon 16 skipping.³² Future studies directed toward understanding the role of the ErbB2ΔEx16 form in development and tumorigenesis will shed important insight into targeting this key oncogenic driver.

MATERIALS AND METHODS

RNA-seq analysis of breast cancer cell lines

Whole-transcriptome shotgun sequencing (RNAseq) was completed on breast cancer cell lines and expression analysis was performed with the ALEXA-seq software package (Vancouver, BC, Canada) as previously described.³³ Briefly, this approach comprises (i) creation of a database of expression and alternative expression sequence 'features' (genes, transcripts, exons, junctions, boundaries, introns and intergenic sequences) based on Ensembl gene models, (ii) mapping of short paired-end sequence reads to these features, (iii) identification of features that are expressed above background noise while taking into account locus-by-locus noise, (iv) identification of features that are differentially expressed in samples and (v) identification of the subset of differentially expressed features that correspond to alternative expression of mRNA isoforms. RNAseq data were available for 59 lines. An average of 71.0 million (76-bp paired-end) reads passed quality control per sample. Of these, 54.0 million reads mapped to the transcriptome on average, resulting in an average coverage of 48.5X across all known genes. Log2 transformed estimates of ErbB2 exon-level and junction-level expression were extracted for analysis. The abundance of ErbB2ΔEx16 was determined using the estimated expression of the E15:E17 junction. Full-length expression was determined by taking the mean of E15, E16 and E17 exons and the E15:E16 and E16:E17 junctions. Error bars represent s.d.

Figure 7. Analysis of transcription factor activation from gene expression profiling. **(a)** Downstream of ErbB2ΔEx16, the transcription factors Smad2, Stat3 and Hif1α are preferentially activated, as evidenced by enrichment in known target gene expression obtained from gene expression profiling. Enrichment plots by GSEA **(b, d)** suggest strong association of Smad2 ($P=0.0019$) and HIF1α ($P=0.014$) with ErbB2ΔEx16 expression. **(c)** Activity of the Stat3 transcriptional networks is also enriched in ErbB2ΔEx16-expressing tumors ($P=0.008$).

Immunoblot analysis

Flash frozen tumor tissue was disrupted by mortar and pestle in liquid nitrogen, and lysed in a modified Hepes lysis buffer (50 mM Hepes pH 7.5 (Bioshop HEP001, Burlington, ON, Canada), 150 mM NaCl (Biobasic SB0476, Markham, ON, Canada), 10% glycerol (Biobasic GB0232), 0.5% NP-40 (Biobasic NDB0385), 1 mM EDTA pH 8.0 (Biobasic EB0185), 10 mM NaF

(Bioshop SFL001), 1 mM PMSF (Bioshop PMS123), 10 mM β glycerophosphate (Bioshop GYP001), 1 mM Na₃VO₄ (Bioshop SOV664), 10 μg/ml aprotinin (Biobasic AD0153), 10 μg/ml leupeptin (Biobasic LDJ691). Lysates were quantified by Bradford assay, and reduced in sodium dodecyl sulfate loading buffer with β-mercaptoethanol (Sigma M6250, St Louis, MO, USA). Immunoblots are representative of three independent experiments.

Antibodies

The following primary antibodies were used for immunoblotting: (Santa Cruz Biotechnology, Dallas, TX, USA) ErbB3 C17 (sc-285), (Cell Signaling Technology, Danvers, MA, USA) phospho-ErbB2 Y1221/1222 (2249), EGFR (2232), phospho-Src family kinase Y416 (2101), phospho-ErbB3 Y1289 (4791), phospho-p38 (9215), p38 T180/Y182 (9212), tubulin (2148), phospho-Smad2 S245/250/255 (3104), Smad2/3 (3102), HIF1α (3716), phospho-Stat3 Y705 (9145), Stat3 (9139), phospho-YB-1 S102 (2900), (Millipore, Billerica, MA, USA) c-ErbB2 Ab-3 (OP-15), (BD Transduction, San Jose, CA, USA) E-cadherin (610182) and (Abcam, Cambridge, UK) YB-1 (12148).

Cell surface biotinylation

Surface receptors were biotinylated with Sulfo NHS ss Biotin (Fisher-Pierce 21331, Waltham, MA, USA) to monitor their rates of internalization. Control cells were either not incubated at 37 °C (0-min control) or incubated for 3 h at 37 °C without subsequent incubation in the biotin stripping buffer (total surface receptor control). Cells were lysed in PLCγ lysis buffer. Biotinylated protein was precipitated from 500 g of total lysate with streptavidin-coated agarose beads (Fisher-Pierce 20347) overnight at 4 °C. The lysate was then washed and prepared for immunoprecipitation as described previously, and analyzed by immunoblot using ImageQuant TL software (GE Healthcare Biosciences, Pittsburgh, PA, USA). The experiment was repeated three times, and representative results are shown. Error bars represent s.d.

RPPA of transgenic mammary tumors

Flash frozen tumor pieces were disrupted using a mortar and pestle while immersed in liquid nitrogen, and transferred to a 50 ml conical tube. Once nitrogen has evaporated, tissue was lysed in NP-40 Lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EDTA, 10 mM NaF, 1 mM PMSF, 10 mM β glycerophosphate, 1 mM Na₃VO₄, 10 μg/ml aprotinin, 10 μg/ml leupeptin) on ice for 10 min. For RPPA analysis, cellular proteins were denatured by 1% sodium dodecyl sulfate (with β-mercaptoethanol) and diluted in five twofold serial dilutions in dilution buffer (lysis buffer containing 1% sodium dodecyl sulfate). Serial diluted lysates were arrayed on nitrocellulose-coated slides (Grace Biolab, Bend, OR, USA) by Aushon 2470 Arrayer (Aushon BioSystems, Billerica, MA, USA) and subjected to RPPA as described previously.³⁴ Analysis of the RPPA data was performed by pooling the sample data by genotype ($n = 5$ for MMTV/ErbB2, $n = 10$ for ErbB2ΔEx16), and statistical significance was determined by subjecting the means to an unpaired Student's *T*-test, with a *P*-value cutoff of < 0.05 . Error bars represent s.d.

Genotyping

Transgenic mice were genotyped using the following primers for MTB (forward 5'-ACCGTACTCGTCAATTCGAAGG-3', reverse 5'-TGCGCCATTATTA CGACAAGC-3'), enhanced green fluorescent protein for ErbB2ΔEx16 (forward 5'-CACAAGTTCAGCGTGTCC-3', reverse 5'-TGACAGCTCGTCCATGC-3') and MMTV-ErbB2 (forward 5'-CCTCCTAAAGGACCTAGAGGAAGGC-3', reverse 5'-CAAGGCCAGGAGAGGCACTGGGGAG-3').

Generation of transgenic mice

A DNA fragment containing the Tet-responsive promoter and *erbB2ΔEx16* cDNA was generated by *PvuII* restriction digest, and sent for pronuclear injection by the McGill LSC Transgenic Core Facility. Putative founder lines were interbred with transgenic mice harboring the MMTV/reverse tetracycline transactivator transgene (MTB, a kind gift from Dr Lewis Chodosh). Bigenic female mice were induced for 5 days with 2 mg/ml doxycycline in their drinking water. Transgene expression was assessed by immunoblot analysis using the thoracic mammary gland, and by whole-mount enhanced green fluorescent protein and hematoxylin staining of the abdominal mammary gland. MMTV/ErbB2 mice were a generous gift from Genentech (Roche, Basel, Switzerland).¹⁸ Female transgenic mice carrying the MTB and ErbB2ΔEx16 transgenes were induced at 8–10 weeks of age with 2 mg/ml doxycycline (Wisent 450-185-EG, St Bruno, QC, Canada) in the drinking water, with tumor onset monitored by weekly palpation. Statistical significance was determined using a Rank Log (Mandel–Cox) test ($n = 25$ for MMTV/ErbB2, $n = 33$ for ErbB2ΔEx16).

Histological staining

Tumor pieces were fixed in 10% neutral buffered formalin for 24 h, processed and embedded in paraffin at the McGill LSC Histology core facility. Sections were cut and stained with Harris' hematoxylin and eosin, or Gomori's trichrome Stain kit (Polysciences 24205-1, Warrington, PA, USA) according to the manufacturer's protocol. Images are representative of at least 10 independent samples. For immunohistochemistry, cells were treated as described previously,³⁵ using Signalstain ABC kit (Vector Laboratories PK-4000, Burlingame, CA, USA) secondary antibody and liquid DAB+ substrate chromogen system (Dako 8059 S, Carpinteria, CA, USA) to develop, as per the manufacturer's instructions. Primary antibodies were anti-keratin 14 (1:500, Covance, cat # PRB-155P, Pinceton, NJ, USA), anti-keratin 5 (1:500 Covance, cat # PRB-160P), anti-keratin 6 (1:500, Covance, cat # PRB-169P) anti-keratin 8/18 (1:200, Fitzgerald, cat # RDI-PROGP11, Acton, MA, USA), CD3ε (1:200, Abcam, Ab16669), phospho-Stat3 Y705 (1:200, Cell Signaling Technology, #9145). Stained sections were quantified using a nuclear staining algorithm on the Aperio Imagescope software (Leica Biosystems, Wetzlar, Germany). All processed samples were single-blinded for genotype during experiment and analysis. Statistical significance was determined by two-tailed, unpaired, Student's *T*-test (CD3ε; $n = 10$, pStat3; $n = 5$). Error bars represent s.d. For immunohistochemistry, fluorescent secondary antibodies Alexa488, Alexa555, and Alexa647 (1:1000, Molecular Probes, Waltham, MA, USA), and counterstained using 4,6-diamidino-2-phenylindole (Sigma, 32670).

Lung metastasis

At necropsy, lung tissue was fixed and processed similarly to tumor pieces. Formalin-fixed, paraffin-embedded lung samples were step sectioned to obtain five sections at 50 μm intervals, and stained with Harris' hematoxylin and eosin. Samples were blinded for genotype, and metastases were counted by microscopic inspection ($n = 18$ for MMTV/ErbB2, and $n = 22$ for ErbB2ΔEx16). Metastatic burden analysis was performed by first determining whether the populations presented with different variances using an *F*-test. As the variances of the two groups were found to be significantly different, evaluation of significance of the means was determined using an unpaired Welch's *T*-test. Error bars represent s.d.

RNA extraction and microarray analysis

Total RNA from transgenic tumor samples ($n = 9$ for MMTV/ErbB2, $n = 7$ for ErbB2ΔEx16) was harvested using the RNeasy mini kit (Qiagen 74106, Hilden, Germany) according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA) and its integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). Cyanine 3-labeled CTP cRNA was produced with 1 μg of total RNA using the Low Input Quick Amp Labeling Kit, according to the manufacturer's instructions (Agilent Technologies, Inc.). The labeled cRNA was then normalized at 600 ng, fragmented and hybridized on SurePrint G3 Mouse GE 8x60K. The arrays were incubated in an Agilent Hybridization oven at 65 °C for 17 h at 10 r.p.m. They were washed and scanned on an Agilent DNA Microarray Scanner C. All these steps were done according to Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Inc.). Data were obtained using the Agilent Feature Extraction software version 11.0.1.1. Using the LIMMA R package,³⁶ normalization was performed through the application of background correction with the half setting,³⁷ and subsequent quantile between-array normalization.³⁸ Probe data were collapsed to RefSeq features by taking the median value. Unsupervised hierarchical clustering was accomplished through the use of Ward's algorithm using the Pearson correlation distance metric applied on the features having the top 2% highest variance across samples. Array data from ErbB2 samples ($n = 9$) and ErbB2ΔEx16 ($n = 7$) samples were analyzed using the SAM³⁹ algorithm to identify significant fold changes upregulated and downregulated in the ErbB2ΔEx16 samples as compared with the ErbB2-positive samples. The top 1000 differentially regulated genes were placed into iRegulon⁴⁰ with Smad2, STAT3 and Hif1α to view gene interactions between differentially regulated genes and Smad2, STAT3 and Hif1α. Gene set enrichment analysis⁴¹ was performed to identify Smad2 and Hif1α gene set enrichment in the ErbB2ΔEx16 samples using genePattern. Stat3 activity was predicted using a gene signature approach according to previous studies.^{42–44} Immune gene signature was determined by subjecting the processed gene signatures to Enrichr gene enrichment analysis.⁴⁵

Cell culture

NMuMG (ATCC CRL1636, Manassas, VA, USA) and SKBR3 (ATCC HTB30) cells were cultured in Dulbecco's modified Eagle's medium (Wisent 319-005-CL) supplemented with 10% fetal bovine serum (Wisent 095-150), 10 mM HEPES pH 7.5 (Wisent 330-050-EL), 10 µg/ml insulin (Wisent 511-016-UG), 100IU/ml penicillin and 100 µg/ml streptomycin (Wisent 450-201-EL). NMuMG-ErbB2wt and NMuMG-ErbB2ΔEx16 cell lines were transfected with either pMSCV-ErbB2wt or pMSCV-ErbB2ΔEx16 plasmids using Genejuice (Novagen 70967, Waltham, MA, USA), and selected with 2 µg/ml puromycin (Clontech 631305, Mountain View, CA, USA). NMuMG-NeuNT cells were generated as reported previously.¹⁵ For doxycycline-inducible cell lines, before pMSCV transfection, NMuMG cells were first infected with lentivirus carrying pTIBZ-ErbB2wt or pTIBZ-ErbB2ΔEx16, selected in 8 µg/ml blasticidin (Thermo Fisher A1113903, Waltham, MA, USA), induced with 10 µg/ml doxycycline, and sorted by fluorescence-activated cell sorting analysis for robust ErbB2 expression. All cell lines were negative for mycoplasma.

Reverse transcriptase–PCR analysis

One microgram of total RNA was converted to cDNA using the NEB ProtoScript II First Strand cDNA Synthesis Kit (New England Biolabs E6560S, Ipswich, MA, USA). Quantitative–PCR reactions were performed in triplicate using 1/10 of the RT product with the Roche LightCycler 480 SYBR Green I Master kit (Roche 04707516001). ErbB2ΔEx16 mRNA was detected using specific primers (forward 5'-CAGCGGTGTGAAACCTGACC-3', reverse 5'-TG GACGTGACAGGGGAGTGG-3'), normalized to either wild-type ErbB2 (forward 5'-GTGGACCTGGATGACAAAGG-3', reverse 5'-TGCTGCCGTGCTTGA TGAG-3') or glyceraldehyde 3-phosphate dehydrogenase (forward 5'-G TGGTCTCTGACTTCAAC-3', reverse 5'-GTTGCTGTAGCCAAATTCGTTG-3'). All samples were analyzed in triplicate with error bars representing s.d.

Orthotopic injections

Experiments were conducted according to an approved institutional Animal Use Protocol. In all, 1x10⁵ cells were injected into the left inguinal fat pad of athymic nude mice (Charles River Laboratories International, Wilmington, MA, USA), with mice fed 2 mg/ml doxycycline in the drinking water, changed weekly. Tumors were monitored by weekly measurements. When tumors reached an approximate size of 1 cm³, doxycycline treatment was withdrawn, and tumors were monitored twice weekly for outgrowth. The results are representative of three repetitions, with *n* = 5 injections per cell line.

Immunofluorescence

In all, 2.5 × 10⁴ cells were seeded on coverslips and allowed to grow for 48 h. Cells were incubated with 100 µg/ml of cycloheximide (Sigma-Aldrich C1988-1G, St Louis, MO, USA) in serum-free media for 1 h. Immunofluorescence was performed as described in Marcotte *et al.*⁴⁶ with the primary antibodies c-ErbB2 (1:500, Dako A048529) and EEA1 (1:100, Santa Cruz Biotechnology sc-6415). Cells were counterstained with 4,6-diamidino-2-phenylindole (Invitrogen D1306, Carlsbad, CA, USA). Confocal images were obtained using an Axiovert 200M microscope (Carl Zeiss Microimaging, Inc., Oberkochen, Germany) and analyzed using LSM5 Image browser (Empix Imaging, Mississauga, ON, Canada). Images are representative of three replicates.

MTT assay

A total of 3000 cells (SKBR3) or 5000 cells (NMuMG and ErbB2ΔEx16 transgenic breast cancer cell lines) were plated in a final volume of 0.2 ml in 96-well flat bottom plates with indicated concentrations of Trastuzumab-DM1. After 3 days, 20 µl of a 5 mg/ml MTT solution in phosphate-buffered saline were added to each well for 4 h. After removal of the medium, 100 µl of dimethylsulfoxide were added to each well. The absorbance at 540 nm was determined using a Bio-Rad Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). At minimum, triplicate wells were assayed for each condition.

Study approval

All animal studies were carried out in accordance with the guidelines of the approved Animal Use Protocol by the McGill University Animal Care Committee (UACC) and the Canadian Council on Animal Care (CCAC).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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