PTEN Deficiency in a Luminal ErbB-2 Mouse Model Results in Dramatic Acceleration of Mammary Tumorigenesis and Metastasis*[^1]

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Signaling pathways.

Overexpression and/or amplification of the ErbB-2 oncogene as well as inactivation of the PTEN tumor suppressor are two important genetic events in human breast carcinogenesis. To address the biological impact of conditional inactivation of PTEN on ErbB-2-induced mammary tumorigenesis, we generated a novel transgenic mouse model that utilizes the murine mammary tumor virus (MMTV) promoter to directly couple expression of activated ErbB-2 and Cre recombinase to the same mammary epithelial cell (MMTV-NIC). Disruption of PTEN in the mammary epithelium of the MMTV-NIC mouse model system dramatically accelerated the formation of multifocal and highly metastatic mammary tumors, which exhibited homogenous pathology. PTEN-deficient/NIC-induced tumorigenesis was associated with an increase in angiogenesis. Moreover, inactivation of PTEN in the MMTV-NIC mouse model resulted in higher levels of the phosphatidylinositol 3′-kinase/Akt signaling pathway. However, like the parental strain, tumors obtained from PTEN-deficient/NIC mice displayed histopathological and molecular features of the luminal subtype of primary human breast cancer. Taken together, our findings provide important implications in understanding the molecular determinants of mammary tumorigenesis driven by PTEN deficiency and ErbB-2 activation and could provide a valuable tool for testing the efficacy of therapeutic strategies that target these critical signaling pathways.

Genetic alterations in the normal mammary epithelium, including the activation of oncoproteins and the loss of function of tumor suppressor genes, have been implicated in the induction of mammary tumors. ErbB-2 (Neu/HER2) is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which also includes EGFR, ErbB-3 (HER3), and ErbB-4 (HER4) (1). Amplification/overexpression of ErbB-2 has been observed in 20–30% of human breast cancers, correlating with poor patient prognosis (2, 3). The importance of ErbB-2 in mammary tumorigenesis has been demonstrated by several transgenic mouse models (4), where engagement of the ErbB-2 receptor results in the recruitment of adaptor proteins, activating primarily the Ras pathway (5). ErbB-2–induced mammary tumors exhibit elevated ErbB-3 protein levels (6). ErbB-3 mediates activation of the phosphatidylinositol 3′-kinase (PI3K)/Akt pathway by recruiting the p85 regulatory subunit of PI3K (7, 8). Additionally, the ErbB-2/ErbB-3 heterodimer is thought to be the most biologically active and pro-tumorigenic receptor complex (9, 10). These findings suggest that the concerted activation of both Ras and PI3K signaling through this EGFR family heterodimer plays a critical role in mammary tumorigenesis (9, 10).

Growth and survival pathways can also become oncogenic through the disruption of tumor suppressors. Loss of the tumor suppressor phosphatase and tensin homologue deleted on chromosome ten (PTEN) due to mutation, loss of heterozygosity (LOH), and epigenetic down-modulation has been reported in about 50% of human cancers, including breast cancer (11). PTEN, a lipid phosphatase, directly antagonizes the proto-oncogenic PI3K/Akt pathway by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate to generate phosphatidylinositol 4,5-bisphosphate to attenuate Akt activation (12, 13). PTEN-controlled PI3K/Akt signaling regulates various cellular events, including proliferation and survival (14, 15), migration (16, 17), and senescence (18). Furthermore, PTEN-deficiency is associated with resistance to the drug Herceptin (Trastuzumab), a humanized monoclonal antibody targeting ErbB-2 (19). Collectively, these observations suggest that cross-talk between ErbB-2 and PTEN disruption may play a critical role in ErbB-2–induced tumorigenesis.

To directly assess the role of PTEN in ErbB-2–induced mammary tumorigenesis, we recently interbred conditional PTEN

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[^6]: The abbreviations used are: EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3′-kinase; MMTV, murine mammary tumor virus; PTEN, phosphatase and tensin homologue deleted on chromosome ten; LOH, loss of heterozygosity; Gsk3β, glycogen synthase 3β; MIN, mammmary intraepithelial neoplasms; Krt, cytokeratin; Bad, Bcl-associated death promoter; mTOR, mammalian target of rapamycin.
mice to a mouse model expressing a Cre-inducible activated ErbB-2 allele under the transcriptional control of the endogenous ErbB-2 promoter (ErbB-2<sup>2K1</sup>) (20). PTEN deficiency dramatically accelerated ErbB-2-mediated tumorigenesis. About 50% of PTEN-heterozygous/ErbB-2<sup>2K1</sup> tumors displayed LOH at the PTEN locus. PTEN-deficient/ErbB-2<sup>2K1</sup> tumors displayed elevated ErbB-2 protein levels, like the parental strain, but this was not due to genomic amplification or dramatically increased ErbB-2 transcription (20). Strikingly, PTEN-deficient/ErbB-2<sup>2K1</sup> tumors showed morphological heterogeneity with molecular and pathological features of the basal-like and HER2 subtypes of human breast cancer.

One limitation of the PTEN-deficient/ErbB-2<sup>2K1</sup> model is that ErbB-2 expression is driven by the endogenous ErbB-2 promoter (21). Thus, initial ErbB-2 transcription in these mammary epithelial cells is low, and therefore, PTEN inactivation occurs before elevated ErbB-2 levels. To directly evaluate the functional consequences of PTEN disruption in a mouse model that places ErbB-2 under transcriptional control of the strong viral MMTV promoter, we have generated mice co-expressing activated ErbB-2 (6) and Cre recombinase from the same bicistronic transcript, due to an internal ribosome entry sequence placed between the two cDNA sequences (MMTV-NIC) (22). This system couples ErbB-2 and Cre recombinase expression within the same mammary epithelial cell (supplemental Fig. IA). Using this approach we demonstrate that homozygous inactivation of PTEN in the presence of constitutive ErbB-2 expression results in a dramatic acceleration of mammary tumor onset in MMTV-NIC mice. PTEN-deficient/NIC tumor cells exhibit increased capacity to colonize the lung, correlating with elevated angiogenesis in the primary tumors. Furthermore, over 50% of PTEN heterozygous/NIC tumors displayed LOH at the PTEN locus. In contrast to the morphological heterogeneity of the PTEN-deficient/ErbB-2<sup>2K1</sup> tumors, PTEN loss in MMTV-NIC mice results in mammary tumors with a homogenous morphology resembling the parental strain. Unlike the PTEN-deficient/ErbB-2<sup>2K1</sup> model, PTEN-deficient/NIC mammary tumorigenesis is associated with the loss of basal/myoepithelial markers, leading to rapid formation of invasive tumors with histopathological and molecular characteristics of the luminal subtype. Biochemical analysis revealed that loss of PTEN in MMTV-NIC tumors results in hyperactivation of the PI3K/Akt pathway. Consequently, this novel ErbB-2 mouse model may provide valuable insights toward understanding the molecular determinants in mammary tumor progression and represents an attractive tool for testing the efficacy of therapeutic strategies directed against the PI3K/Akt network in combination with receptor tyrosine kinase inhibitors.

**EXPERIMENTAL PROCEDURES**

**Animal Husbandry and Genotyping**—Mice harboring a bicistronic transcript expressing an oncogenic Neu allele (6) followed by an internal ribosome entry sequence element and Cre recombinase under the control of the MMTV promoter have been described (22). These mice were interbred with Flox-PTEN mice (129/J, The Jackson Laboratory, Bar Harbor, ME) to generate bigenic mice, PTEN<sup>+/−</sup>/NIC and PTEN<sup>−/−</sup>/NIC. Genotyping was carried out by PCR as described (21, 23). Nulliparous females were monitored weekly for mammary tumor formation by physical palpation. All procedures involving mice were conducted in accordance with McGill University Animal Care guidelines.

**Histological Analysis**—Mammary tumors and lungs were harvested from mice that were tumor-bearing for 4 weeks. Tissue was fixed and embedded as described (5). Paraffin sections of 5 μm were stained with hematoxylin and eosin. Step sections of lungs (4 × 50 μm) were scanned using a Scanscope XT Digital Slide Scanner (Aperio) and analyzed at 5× magnification using Imagescope software (Aperio) to quantify the total number of lesions per lung. The data are shown as the average number of lesions per lung lobe. Immunohistochemical staining was performed as described (24). Sections were incubated with the primary antibodies Neu (Homemade, 1:100), PTEN (Cell signaling 9559, 1:100), cytokertatin-6 (Krt6; Covance PRB-169P, 1:1000), Krt5 (Covance PRB-160P, 1:1500), Krt14 (Covance PRB-115P, 1:1000), Krt8 (Fitzgerald RDI-PROGP11, 1:1000), smooth muscle actin (Sigma A2547, 1:1000), and Ki67 (Abcam ab15580, 1:100) followed by incubation with Elite anti-mouse, anti-rabbit, or anti-guinea pig IgG secondary antibodies from the Vectastain kit (PK-6102, Vector Laboratories, Burlingame, CA). CD31 staining of was performed on optimal cutting temperature-embedded sections (BD Biosciences 550274, 1:200) as described (25). Slides were scanned using a ScanScope XT Digital Slide Scanner, and data were analyzed with Imagescope software using positive pixel count or nuclear algorithms.

**Immunoprecipitation and Immunoblotting**—Tumor lysates were prepared as described (6). ErbB-2 and ErbB-3 immunoprecipitations were performed with 500 μg of lysate using the ErbB-2 (Ab4) monoclonal antibody (Oncogene Research Products, Inc.) and ErbB-3 (C17) polyclonal antibody (Santa Cruz Biotechnology, Inc.), respectively. Immunoblot analyses were performed on 20 μg of lysate as described (6, 26) using the following antibodies: ErbB-2 (Santa Cruz C18, 1:1000), ErbB-3 (Santa Cruz C17, 1:1000), phosphotyrosine (BD Biosciences PY20, 1:1000), PTEN (Cell Signaling 9559, 1:1000), Akt (Cell Signaling 9272, 1:1000), phospho-Akt (Cell Signaling 9275, 1:1000), Erk (Cell Signaling 9102, 1:1000), phospho-Erk (Cell Signaling 9101, 1:1000), Tsc2 (Cell Signaling 3612, 1:1000), phospho-Tsc2 (Cell Signaling 3611, 1:1000), Bcl-associated death promoter (Bad; BD Transduction Laboratories 36420, 1:1000), phospho-Bad (Cell Signaling 5286, 1:1000), glycogen synthase 3B (Gsk3β; Cell Signaling 9315, 1:1000), phospho-Gsk3β (Cell Signaling 9331, 1:1000), and Actin (Sigma A-9718, 1:1000). Horseradish peroxidase-conjugated secondary antibodies (1:10000) were obtained from The Jackson Laboratory.

**DNA Extraction and Southern Blot**—DNA was obtained from tumors and examined for LOH at the PTEN locus as described (21).

**DNA Microarray**—RNA sample preparations were performed as previously described (27). Total RNA was isolated from five individual (PTEN<sup>−/−</sup>/NIC) samples. Two RNA pools containing equal amounts of total RNA from five individual MMTV-NIC tumors were generated and functioned as a biological repeat. Labeled aRNA (750 ng) was hybridized against a Universal Mouse Reference RNA (Stratagene) onto Agilent Whole Mouse Genome Oligo Microarrays (G4122A; 44K).
Duplicate hybridizations were done for all samples using reverse-dye labeling.

Microarray Data Analysis—All microarray data analysis was carried out in the R statistical programming environment (R Development Core Team, 2008) with Bioconductor (28). Gene expression data preprocessing and normalization of in-house datasets was carried out as described (27). Raw and normalized data were uploaded to the NCBI Gene Expression Omnibus data base (GEO) and is accessible as data series GSE13916 (ncbi.nlm.nih.gov). Murine and human datasets from Herschkowitz et al. (44) were obtained from GEO and normalized in the same manner as in the original publication. Class distinction was performed using limma (29). Overrepresented GO and KEGG categories were computed by means of a standard hypergeometric test on the set of genes overexpressed in a group of samples and was carried out using the GOstats package (30). For the Gene Set Enrichment Analysis (GSEA), genes were ranked using the program’s built-in algorithm, with 1000 permutations (31, 32). Symbols in all gene sets were translated from human to mouse using biomaRt (33). All unsupervised hierarchical clustering was performed using the Pearson correlation as a distance measure and with Ward’s minimum variance agglomeration method. Integration of separate datasets was made by subtracting log2 ratios from normal tissue and scaling each gene to \( N(0,1) \) within and across all samples within the dataset.

RESULTS

Heterozygous or Homozygous Loss of PTEN Function Accelerates ErbB-2-induced Mammary Tumorigenesis and Metastasis—We recently generated a mouse model where expression of activated ErbB-2 and Cre recombinase are coupled within the same mammary epithelial cell (22). To determine the biological significance of heterozygous or homozygous PTEN loss in MMTV-NIC-induced mammary tumorigenesis, we interbred MMTV-NIC animals with mice carrying one or two floxed PTEN alleles (PTEN\(^{+/+}\)/NIC, PTEN\(^{+/−}\)/NIC, and MMTV-NIC). Thus, both ErbB-2 expression and loss of PTEN will occur simultaneously in each mammary epithelial cell in these bigenic animals. Cohorts of PTEN\(^{+/+}\)/NIC, PTEN\(^{+/−}\)/NIC, and MMTV-NIC virgin females were generated and monitored for mammary tumor formation by weekly palpation. Homozygous inactivation of PTEN resulted in dramatic acceleration of tumor onset with an average latency of 43 days, whereas heterozygous PTEN loss exhibited a moderate acceleration (126 days) compared with
that of 198 days obtained for the parental strain (Fig. 1A, left panel; Table 1). All genotypes developed multifocal mammary tumors with 100% penetrance (Table 1). Four weeks after initial tumor detection, all tumor-bearing animals were sacrificed, the total tumor volume was determined, and lungs were harvested. The average total tumor burden was not significantly elevated in the PTEN-deficient/NIC mice relative to the parental strain (56%) (Fig. 1 right panel). Interestingly, we found a significant increase in the average number of lung lesions in the PTEN-deficient/NIC mice (53%) and the parental strain (56%) (Fig. 1B, left panel). Interestingly, we found a significant increase in the average number of lung lesions in the PTEN-deficient/NIC mice (2 metastases/lung lobe) relative to the parental strain (0.6 metastases/lung lobe) (Fig. 1B, right panel).

Histological assessment of PTEN-deficient/NIC-derived mammary tumors revealed a morphology similar to that observed in MMTV-NIC tumors. Unlike the morphological heterogeneity observed in the PTEN-deficient/ErbB-2KI mouse model (20), the PTEN-deficient/NIC-derived tumors are composed of large nodular nests with central necrosis that resemble the parental tumors (Fig. 1C).

Moreover, a histological survey of adjacent mammary glands from tumor-bearing mice revealed that 81% of the MMTV-NIC adjacent mammary gland structures represent relatively normal ducts/acini, whereas 1% categorize as early mammary intraepithelial neoplasms (MINs), 8% as hyperplasia and 10% as ectasia (supplemental Fig. 1C). In comparison, PTEN-deficient/NIC adjacent mammary glands contained only 38% relatively normal ducts/acini, 8% early MINs, 20% hyperplasia, and 34% ectasia, suggesting that PTEN inactivation may result in increased numbers of pre-neoplastic structures associated with early stages of tumorigenesis (supplemental Fig. 1C). Collectively, these observations suggest that impairment of PTEN has a dramatic impact on both tumor initiation and metastatic potential in ErbB-2-mediated mammary tumorigenesis.

### TABLE 1

Phenotypic characteristics of the PTEN-deficient/ErbB-2 mouse models

| Strain | Promoter (activated ErbB-2) | Average age of mammary tumor formationa | Penetration of tumorsb | Mammary tumor morphology | % of animals with lung lesionsd | % of mammary tumors with PTEN lossd |
|--------|-----------------------------|----------------------------------------|------------------------|--------------------------|-------------------------------|----------------------------------|
| PTEN+/−/NIC | MMTV | 126 (±36) | 100 | Multifocal, solid nodular | 53 (10/19) | 80 |
| PTEN+/−/NIC | MMTV | 43 (±12) | 100 | Multifocal, solid nodular | 87.5 (14/16) | 100 |
| MMTV-NIC | MMTV | 198 (±43) | 100 | Multifocal, solid nodular | 56 (5/9) | ND |
| PTEN+/−/ErbB-2KI (20) | Endogenous | 212 (±92) | 100 | 50% focal, 50% multifocal heterogeneous morphology | 35 (6/17) | 56 |
| PTEN+/−/ErbB-2KI (20) | Endogenous | 138 (±125) | 100 | 60% focal, 40% multifocal heterogeneous morphology | 0 (0/6) | 0 |
| ErbB-2KI (21) | Endogenous | 419 (±137) | 83 | Focal comedo adenocarcinoma | 6 (2/35) | 0 |
| PTEN+/− (20) | NA | 410 (±101) | 75 | 60% focal, 40% multifocal heterogeneous morphology | 18 (2/11) | 17 |
| PTEN+/− (20) | NA | NA | NA | NA | NA | NA |

* a Values represent the average age of onset in days of first palpable mammary tumor.
* b Percentage of animals that developed mammary tumors.
* c Percentage of tumor-bearing animals that possessed lung lesions; parentheses indicate the numbers of animals with lung lesions over the number of examined animals.
* d Percentage of tumor-bearing animals showing a reduced amount of PTEN by Southern blot analysis.

PTEN in MMTV-ErbB-2 Mammary Tumorigenesis

PTEN-deficient/NIC-mediated Mammary Tumor Progression Is Associated with Increased Angiogenesis—Given that PTEN inactivation in our MMTV-driven ErbB-2 mouse model has a significant impact on mammary tumor initiation (Fig. 1A), we next determined whether tumor cell proliferation was affected. Serial tumor sections were subjected to immunohistochemical staining with the proliferative marker Ki67. This survey revealed that PTEN-deficient tumors possessed similar proliferative levels compared with their wild type counterparts (Fig. 2A). However, analysis of adjacent mammary glands from both genotypes displayed a moderate increase in Ki67-positive nuclei within early MINs and hyperplasia of the PTEN-deficient/NIC mice when compared with the parental strain (Fig. 2A).

One interesting feature of PTEN-deficient mammary tumors is their increased capacity to metastasize to the lung (Fig. 1B, right panel). To determine whether the enhanced metastatic phenotype was related to increased angiogenesis, we performed CD31 immunohistochemical staining to score for blood vessels on mammary tumors from each genotype. Microvessel density is reflected by the percentage of CD31-positive endothelium per area of tumor epithelium and can be used as an indicator of increased angiogenesis. Immunohistochemical examination of CD31 staining revealed that tumors obtained from PTEN-deficient/NIC females displayed significantly increased angiogenesis relative to MMTV-NIC-derived tumors (Fig. 2B). Our findings suggest that the dramatic effect of PTEN inactivation on ErbB-2-mediated mammary tumorigenesis and metastasis may be due to increased tumor angiogenesis.

MMTV-NIC-dependent Mammary Tumor Progression Is Associated with Loss of Basal/Myoepithelial Markers—Histopathological surveys of PTEN-deficient/NIC and MMTV-NIC adjacent mammary glands revealed that early MINs, hyperplasia, and relatively normal ducts/acini, stained intensely for basal/myoepithelial markers including smooth muscle actin, Krt5, Krt14 (34–36), and Krt6, a cell differentiation marker (37) (supplemental Figs. 2 and 3). Expression of these markers inversely correlated with the pattern of ErbB-2 staining in early MINs and hyperplasia (Fig. 3, supplemental Fig. 4).
In contrast, end-stage PTEN-deficient/NIC and MMTV-NIC tumors exhibited strong ErbB-2 expression accompanied by the absence of these basal/myoepithelial markers. Additionally, tumors from both genotypes primarily expressed the luminal marker Krt8 (38).

We further assessed whether PTEN-deficient/NIC- and MMTV-NIC-derived lung metastases resemble primary tumor characteristics by subjecting them to comprehensive immunohistochemical analyses. Lung lesions from both strains strongly expressed ErbB-2 and Krt8, whereas PTEN was only detected in MMTV-NIC mice. Krt5 or Krt6 expression was not observed in either of the strains (supplemental Fig. 5). PTEN<sup>−/−</sup>/NIC-derived metastases displayed higher but variable Krt14 expression and a moderate increase in proliferation relative to the parental MMTV-NIC metastatic lesions (supplemental Fig. 6A and B).

Interestingly, a number of tumor cells in the PTEN-deficient/NIC metastases expressed both Krt14 and Krt8, which may suggest that these cells belong to an undifferentiated precursor population (supplemental Fig. 6C). Collectively, these data suggest that tumor progression in both strains is associated with the loss of basal/myoepithelial markers, which is accompanied by increased ErbB-2 expression.

PTEN-deficient/NIC-mediated Mammary Tumor Progression Is Associated with LOH at the PTEN Locus and Activation of the MAPK and PI3K/Akt Pathways—Although homozygous PTEN disruption in MMTV-NIC mammary glands dramatically reduced tumor latency, inactivation of only one PTEN allele was sufficient to accelerate tumor initiation compared with the parental strain (Fig. 1A). To ascertain whether tumor progression was associated with loss of the remaining PTEN allele, we performed Southern blot analyses on tumor-derived genomic DNA with PTEN-specific probes that distinguish between the conditional and wild type PTEN alleles. More than 50% of the examined tumors displayed loss of their wild type PTEN allele (Fig. 4A, Table 1). This observation corresponded with reduced PTEN protein levels in comparison to the MMTV-NIC strain, as determined by either immunoblot or immunohistochemical analyses (supplemental Fig. 7A). The low PTEN protein levels that were evident in PTEN-deficient samples likely represent stromal tissue that retained PTEN alleles. Indeed, immunohistochemical analyses of PTEN-deficient/NIC tumors revealed PTEN expression in myoepithelial and endothelial cells (supplemental Fig. 7B) (39, 40). These data indicate that LOH of the remaining wild type PTEN allele is frequently noted in PTEN<sup>−/−</sup>/NIC-induced mammary tumors.

To ensure that PTEN-deficient/NIC tumors express activated ErbB-2, we subjected ErbB-2 immunoprecipitates from
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mammary tumor lysates to immunoblot analysis using ErbB-2 and phosphotyrosine antibodies. High ErbB-2 levels and ErbB-2 activation were confirmed in all tumors (Fig. 4B, upper panel). To assess whether PTEN-deficient/NIC tumors are capable of activating ErbB-2 downstream targets, such as the MAPK signaling pathway (5), tumor lysates were immunoblotted for Erk and phospho-Erk. Like the MMTV-NIC model, PTEN-deficient/NIC tumors expressed similar levels of activated Erk (Fig. 4B, lower panel), indicative of efficient activation of this pathway.

Given that PTEN functions as a negative regulator of the PI3K/Akt pathway, its loss can lead to hyperactivation of Akt (13). Therefore, we examined PTEN-deficient/NIC mammary tumor lysates by immunoblot analysis using Akt and phospho-Akt antibodies. As predicted, loss of PTEN function increased Akt phosphorylation relative to the parental strain (Fig. 4C, upper panel), indicating amplification of the PI3K/Akt signal in PTEN−/−/NIC mice. Thus, we investigated the activation of PI3K/Akt downstream targets, including tuberin (Tsc2), Bad, Gsk3β, and the mammalian target of rapamycin (mTOR). Immunoblot analysis revealed that the majority of PTEN−/−/NIC tumors increased phosphorylation of three direct Akt targets, Tsc2, Gsk3β, and Bad (41–43) (Fig. 4D). However, phosphorylated mTOR levels varied in tumors derived from both mouse strains, which correlated with total mTOR protein (data not shown). Together, these data provide evidence that PTEN inactivation in MMTV-NIC-induced mammary tumorigenesis gives rise to tumors that have acquired hyperactivation of the PI3K/Akt pathway.

**PTEN−/−/NIC and MMTV-NIC Mouse Models Display Molecular Characteristics of the Luminal Subtype of Breast Cancer**—Our histological and biochemical analyses suggest that disruption of PTEN function in MMTV-NIC-driven mammary tumorigenesis engages distinct phenotypic changes in tumor latency, metastasis, angiogenesis, and the activation of the PI3K/Akt pathway (Figs. 1, A and B, 2B, and 4C). To elucidate the molecular basis of these events, we subjected RNA from PTEN−/−/NIC and MMTV-NIC tumors to global transcriptional profiling. Class distinction analysis was applied to identify strain-specific transcriptional changes. However, only 244 genes were found to be differentially expressed between the two tumor types (p value ≤0.05; supplemental Table 1). Of these, four genes (von Willebrand factor A domain containing 1, vwa1; andrenomudellin, adm; tenomodulin, tnmd; Eph receptor A2, epha2) are implicated in angiogenesis and/or cell migration and were significantly overexpressed in the PTEN-deficient model and may reflect the increased microvessel density and metastatic potential in these tumors (Figs. 1B and 2B).

Pathway analysis between the PTEN−/−/NIC and MMTV-NIC tumors revealed few biologically relevant gene sets that were significantly associated with one versus the other genotype. When separately comparing the transcriptional profiles of either mouse model to normal mammary gland-derived profiles, a number of pathways were found to be significantly associated with each tumor group. However, the vast majority of these categories were common to both genotypes again, suggesting that tumors from both strains exhibit strikingly similar molecular features (supplemental Table 2).

Given that the PTEN-deficient/NIC- and MMTV-NIC-derived mammary tumors display similar luminal-like morphology (Fig. 1C), we next compared their transcriptional

| A | PTEN+/NIC | MMTV-NIC | PTEN+/NIC |
|---|-----------|-----------|-----------|
| IP: Neu | 2128 | 963 | 951 |
| IB: Neu | 963 | 951 | 951 |
| IP: Neu | 963 | 951 | 951 |
| IB: p-Tyr | 963 | 951 | 951 |
| IB: Erk | 963 | 951 | 951 |
| IB: p-ERK | 963 | 951 | 951 |
| IB: actin | 963 | 951 | 951 |

**FIGURE 4.** PTEN-deficient/NIC mammary tumorigenesis is associated with LOH at the PTEN locus and activation of the MAPK and PI3K/Akt pathway. **A,** Southern blot analysis of tumor DNA from the indicated genotypes. The detectable bands represent the wild type (WT) and mutant alleles. **B,** ErbB-2 was immunoprecipitated (IP) from tumor lysates (500 μg) and subjected to ErbB-2 and phosphotyrosine immunoblot (IB) analysis. These same lysates (20 μg) were subjected to Erk and phospho-Erk (IB) and Akt and phospho-Akt (C) immunoblot analysis. ErbB-3 immunoprecipitation was performed on 500 μg of tumor lysates followed by ErbB-3 and phosphotyrosine immunoblot analysis. **D,** biochemical examination of PI3K/Akt downstream targets. Tumor lysates (20 μg) were subjected to Tsc2, phospho-Tcs2, Bad, phospho-Bad, Gsk3β, and phospho-Gsk3β immunoblot analysis. p, phosphorylated.
profiles to molecular signatures derived from tumors of various transgenic mouse models, including luminal-like (Neu, Myc, and PyMT models) and basal-like models (Wnt1, Brca1-deficient, and p53-deficient models) (44). Using a murine-specific intrinsic gene set (44), both the PTEN-deficient/NIC and the MMTV-NIC strain clustered closely together within a group consisting of typical luminal-like mouse models, including MMTV-Neu (Fig. 5A). Furthermore, the PTEN-deficient/NIC- and MMTV-NIC-derived transcriptional profiles were compared with those of human breast cancers using a cross-species intrinsic gene set as a basis (44). Both mouse models clustered primarily with the human luminal subtype (Fig. 5B). Overall, these data support our histopathologic observations that end-stage mammary tumors from both genotypes display comparable morphologies with luminal-like characteristics.

**DISCUSSION**

Genetic alterations in both ErbB-2 and PTEN have been observed in human breast cancers (2, 3, 45). Elevated ErbB-2 levels due to increased copy number are observed in 20–30% of human breast cancers and correlate with poor patient prognosis (2, 3). By contrast, PTEN loss has been reported to occur in 5–10% of human breast cancers and has been implicated in the induction of basal-like breast cancers (11, 46, 47). There is also compelling evidence suggesting that disruption of PTEN function may have an impact on ErbB-2-positive human breast cancer. For example, PTEN loss has been implicated in conferring trastuzumab resistance in ErbB2-positive breast cancer cell lines and patient samples (19, 48). Recently, we have demonstrated that PTEN deficiency in transgenic mice expressing activated ErbB-2 under the transcriptional control of its endogenous promoter (ErbB-2KI) results in acceleration of mammary tumor onset (20). Unlike the parental strain, induction of tumors in the PTEN-deficient/ErbB2KI model occurs in the absence of ErbB-2 gene amplification or elevated ErbB-2 transcription. Indeed, like PTEN-deficient human breast cancers, the tumors that arise in this model possess a molecular signature that resembles the human basal subtype (20). These observations suggest that PTEN impairment can cooperate with activated ErbB-2 to accelerate mammary tumorigenesis.

To further elucidate molecular determinants that are important in PTEN-deficient/ErbB-2-induced mammary tumorigenesis, we have generated a novel PTEN-dependent mouse model that co-expresses activated ErbB-2 and Cre recombinase under the transcriptional control of the MMTV promoter (MMTV-NIC strain). Loss of either one or both PTEN alleles resulted in dramatic acceleration of mammary tumor progression (Fig. 1A, Table 1). Although all females developed multifocal mammary tumors, resembling the typical solid nodular ErbB-2-type morphology, the incidence of lung metastases was increased in
PTEN-deficient/NIC animals (Fig. 1B, Table 1). These findings suggested that PTEN-deficient/NIC tumors may exhibit an increase in proliferation and/or angiogenesis. Indeed, when the microvessel density was determined by immunohistochemical analysis in the various genotypes, we obtained a 3-fold increase in CD31-positive cells in the PTEN-deficient/NIC tumors compared with the parental strain (Fig. 2B). Given these results, loss of PTEN function may provide a selective advantage during mammary tumor progression in MMTV-NIC mice, resulting in a highly invasive phenotype.

Like the PTEN-deficient/ErbB-2\(^{KI}\) model system, disruption of only one PTEN allele had a significant impact on mammary tumor onset in the MMTV-NIC strain, and LOH of the remaining wild type PTEN allele occurred in over half of the PTEN-heterozygous/NIC tumors analyzed (Figs. 1A and 4A). Loss of PTEN function through LOH has not only been frequently observed in human breast cancer but also in various PTEN-deficient mouse models, including the PTEN-deficient/ErbB-2\(^{KI}\) model (20, 45, 49–51). Immunohistochemical and biochemical analyses on tumors from PTEN-deficient/NIC mice confirmed loss of PTEN protein expression (supplemental Fig. 7). The variation in the degree of PTEN loss may be due to varying amounts of stromal tissue in the tumors, the efficiency of Cre recombinase, and the extent of LOH at the PTEN locus. Furthermore, the majority of PTEN-deficient/NIC-derived tumors displayed high levels of ErbB-2 expression and activation of this receptor tyrosine kinase (Fig. 4B). Accordingly, Erk (Fig. 4B) as well as p38 (data not shown) were phosphorylated in tumors, confirming activation of the MAPK pathway in the PTEN-deficient/NIC mouse model.

Assessment of target proteins downstream of PTEN revealed elevated Akt phosphorylation and increased phosphorylation of three direct Akt targets, Tsc2, Gsk3\(\beta\), and Bad, in the majority of PTEN-deficient/NIC-derived tumors (Fig. 4, C and D). Phosphorylation of these proteins by Akt is inhibitory, leading to activation of mTOR in the case of Tsc2 (43) and promoting cell survival through Gsk3\(\beta\) and Bad (41). The variability in mTOR protein levels observed (data not shown) indicates that steady state levels of mTOR activation do not appear to be significantly regulated by alteration in PI3K/Akt signaling in this context. These data suggest that PTEN inactivation in MMTV-NIC-induced mammary tumorogenesis hyperactivates PI3K/Akt signaling, which in turn may elicit cellular responses through a range of effector pathways, including cell growth (Tsc2) and survival (Gsk3\(\beta\), Bad). Given the importance of the PI3K/Akt network in the induction of human breast cancer (52), the development of small molecule antagonists for components of this pathway may have an important impact on treating breast cancer progression. In this regard, the PTEN-deficient/NIC transgenic mouse model would be suitable for investigating the efficacy of these reagents in modulating the disease process.

Mammary tumors derived from our PTEN-deficient/NIC mouse models possess a homogeneous morphology that histopathologically resembles the parental strain. Tumors derived from both genotypes were predominantly composed of luminal epithelial cells as indicated by Krt8 immunostaining and the lack of cells expressing basal/myoepithelial markers (supplemental Fig. 2). However, immunohistochemical examinations of adjacent mammary glands revealed the presence of basal/myoepithelial cells in hyperplasia and early MINs (supplemental Fig. 2). Interestingly, the cellular levels of these basal/myoepithelial markers inversely correlated with ErbB-2 expression, indicating that loss of PTEN function may be a critical factor for early mammary tumor initiation in the MMTV-NIC mouse model (Fig. 3). However, upon induction of increasing levels of activated ErbB-2 in luminal mammary epithelial cells during tumor progression, developing tumors transition into an invasive stage indicated by the disappearance of the basal membrane and the myoepithelial layer. Therefore, like the parental MMTV-NIC strain, high ErbB-2 expression and sustained activation may drive PTEN-deficient/NIC mammary tumors toward the luminal subtype. In support of this inference, transcriptional profiling revealed PTEN\(^{-/-}\)/NIC and MMTV-NIC tumors display strikingly similar molecular characteristics that not only reflected luminal-like murine models but also the luminal subtype of primary human breast cancer (Fig. 5). In addition, MMTV-NIC-derived lung metastases displayed characteristics of the corresponding primary tumors. Interestingly, PTEN-deficient/NIC lung lesions exhibited features of the primary tumors (high ErbB2 expression, loss of PTEN, Krt5 and Krt6 expression) but also of hyperplasia/MINs (increased proliferation and Krt14 expression) (Fig. 2A, supplemental Figs. 5 and 6). These observations may argue that loss of PTEN results in dissemination of tumor cells early during tumor progression (53, 54). Further studies are needed to investigate this hypothesis.

In contrast to PTEN-deficient/NIC mice, the PTEN-deficient/ErbB-2\(^{KI}\) mouse model places the oncogene under the control of its endogenous promoter to mimic physiological levels of ErbB-2 during mammary tumorigenesis (21). These mice developed tumors with a remarkable morphological heterogeneity, partly representing features of the basal-like subtype. By contrast, MMTV-NIC-driven mouse models utilize a strong viral promoter to induce ErbB-2 expression, resulting in high ErbB-2 expression much earlier compared with the PTEN-deficient/ErbB-2\(^{KI}\) model (Fig. 5C). As a consequence, the PTEN-deficient/NIC model may confer a predominant ErbB-2 phenotype in tumors that ultimately develop. Although loss of PTEN is crucial for tumor initiation and progression in both the MMTV-NIC and the ErbB-2\(^{KI}\) models, the difference in ErbB-2 protein levels expressed during the early stages of tumorigenesis in these models may influence which tumor cell type is selected during this process. Thorough comparisons of both PTEN-dependent ErbB-2 model systems may result in the identification of signaling networks that will provide potential targets for therapeutic intervention in the treatment of breast cancer.

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