Critical Roles of DMP1 in Human Epidermal Growth Factor Receptor 2/neu-Arf-p53 Signaling and Breast Cancer Development

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Abstract

Human epidermal growth factor receptor 2 (HER2) overexpression stimulates cell growth in p53-mutated cells while it inhibits cell proliferation in those with wild-type p53, but the molecular mechanism is unknown. The Dmp1 promotor was activated by HER2/neu through the phosphatidylinositol-3′-kinase-Akt-NF-κB pathway, which in turn stimulated Arf transcription. Binding of p65 and p52 subunits of NF-κB was shown to the Dmp1 promotor and that of Dmp1 to the Arf promotor on HER2/neu overexpression. Both Dmp1 and p53 were induced in premalignant lesions from mouse mammary tumor virus-neu mice, and mammary tumorigenesis was significantly accelerated in both Dmp1+/− and Dmp1−/− mice. Selective deletion of Dmp1 and/or overexpression of Tbx2/Pokemon was found in >50% of wild-type HER2/neu carcinomas, although the involvement of Arf, Mdm2, or p53 was rare. Tumors from Dmp1+/−, Dmp1−/−, and wild-type neu mice with hemizygous Dmp1 deletion showed significant downregulation of Arf and p21Cip1/WAF1, showing p53 inactivity and more aggressive phenotypes than tumors without Dmp1 deletion. Notably, endogenous hDMP1 mRNA decreased when HER2 was depleted in human breast cancer cells. Our study shows the pivotal roles of Dmp1 in HER2/neu-p53 signaling and breast carcinogenesis. Cancer Res; 70(22); 9084–94. ©2010 AACR.

Introduction

Breast cancer is one of the largest public health issues in the United States and most of the industrialized world (1–4). Breast cancers that are positive for the estrogen receptor (ER) are usually responsive to adjuvant hormonal therapy with antiestrogens and/or aromatase inhibitors and thus have a more favorable prognosis (1). On the other hand, ER-negative breast cancers are often associated with aggressive disease, including amplification of human epidermal growth factor receptor 2 (HER2) or c-Myc oncogenes and mutation of the p53 gene (5). Chemotherapy plus use of the humanized monoclonal antibody to HER2 (trastuzumab) is proven to improve survival of HER2-positive breast cancer patients, but the prognosis of such patients is poor (5).

HER2/neu encodes a receptor type tyrosine kinase that belongs to the epidermal growth factor receptor family (5–9). It is overexpressed in ~30% of breast cancer cases, primarily due to gene amplification. HER2/neu overexpression is found in metastatic lesions and thus is associated with poor prognoses (4–6). Recent studies have stressed the importance of phosphatidylinositol-3′-kinase (PI3K) and serine/threonine kinase Akt/protein kinase B in HER2/neu signaling (10). The PI3K-Akt signaling has also been linked to the induction of NF-κB (11–13). Because transcriptional activation by NF-κB requires its nuclear translocation, degradation of IκB molecules triggered by phosphorylation of serine residues 32/36 by IκB kinases has been considered a key rate-limiting step in NF-κB activation (11). Importantly, both human breast cancer cell lines and clinical specimens often show constitutive activation of NF-κB (14), suggesting oncogenic roles of subsets of NF-κB in breast cancer development.

Dmp1, a cyclin D-binding myb-like protein 1 (also called Dmtf1), was originally isolated in a yeast two-hybrid screen of a murine T-lymphocyte library with cyclin D2 as bait (15). Dmp1 shows its activity as a tumor suppressor by directly binding to the Arf promotor to activate its gene expression and thereby induces Arf- and p53-dependent cell cycle arrest (refs. 16, 17; for Arf reviews, refs. 18, 19). Dmp1-null cells can easily give rise to immortalized cell lines that retain wild-type p19ARF and functional p53 and are transformed by oncogenic Ras alone, suggesting that the activity of the Arf-p53 pathway is significantly attenuated in Dmp1-deficient cells (20, 21).

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The murine Dmp1 promoter is efficiently activated by oncopgenic Ras and is repressed by mitogenic signals mediated by E2Fs and genotoxic signals by NF-κB (refs. 22–24; for review, ref. 25). Both Dmp1\(^{+/−}\) and Dmp1\(^{−/−}\) mice are prone to tumor development when neonatally treated with dimethylbenzanthracene or by ionizing radiation (20, 21). Tumors induced by the Eμ-Myc or K-Ras\(^{−/−}\) transgene were greatly accelerated in both Dmp1\(^{+/−}\) and Dmp1\(^{−/−}\) backgrounds with no differences between groups lacking one or two Dmp1 alleles, suggesting haploid insufficiency of Dmp1 in tumor suppression (21, 26). Moreover, tumors from Eμ-Myc or K-Ras\(^{−/−}\) mice rarely showed p53 mutation or Arf deletion, indicating that Dmp1 is a physiologic regulator of the Arf-p53 pathway in lymphoid and lung epithelial cells (refs. 21, 26; for reviews, refs. 27, 28).

The hDMP1 gene is located on chromosome 7q21, a region often deleted in human breast cancer and hematopoietic malignancies (29–32). We recently found that loss of heterozygosity of hDMP1 was present in ~35% of non–small cell lung carcinomas (26). It was reported that HER2 overexpression in p53 wild-type human ovarian carcinoma cell line became apoptotic shortly after transfection, whereas HER2 expression was associated with cell proliferation in cells with mutated p53 (33). However, the signaling pathway that links HER2 overexpression and activation of p53 has never been shown. Moreover, very little is known about the roles of Dmp1 (or hDMP1) in breast cancer development. The current study was conducted to elucidate the roles of Dmp1 and Arf in HER2/neu signaling and breast carcinogenesis. We show that both Dmp1 and Arf promoters are selectively activated by HER2/neu and both Dmp1 and p53 proteins are induced in precancerous mammary glands from mouse mammary tumor virus (MMTV)-neu mice (34). Of note, the value of MMTV-long terminal repeat (LTR)-driven transgenic mice as models for human breast cancer has recently been reconfirmed because of the discovery of MMTV env-, LTR-like sequences in ~40% of human breast carcinomas (35, 36). We crossed MMTV-neu mice with Dmp1-deficient mice to observe the latency period for tumorigenesis and have conducted extensive molecular genetic analyses of mammary tumors. We also show that human DMP1 is regulated by endogenous HER2 overexpressed in breast epithelial cells.

Materials and Methods

Establishment of Dmp1\(^{+/−}\), Dmp1\(^{−/−}\); MMTV-neu compound mice

Dmp1-heterozygous females were backcrossed to the same FVB/NJ males (The Jackson Laboratory) for more than eight generations to obtain Dmp1\(^{+/−}\) mice with >99% FVB/NJ background overall. One male MMTV-neu (mutant) mouse (The Jackson Laboratory) was crossed with two Dmp1\(^{+/−}\) females to obtain Dmp1\(^{+/−}\); MMTV-neu mice. Then Dmp1\(^{+/−}\); MMTV-neu compound transgenic mice were further crossed with Dmp1\(^{−/−}\) mice to obtain >25 mice with each genetic background. Littermate wild-type mice were used as controls. Mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals.

Real-time PCR

Quantitation of Dmp1, p19\(^{ARF}\), p21\(^{WAF1}\), and p16\(^{Ink4a}\) mRNAs was conducted by real-time PCR Taqman assay by ABI7500 (Applied Biosystems) using β-actin as an internal control (23, 24, 37). For p21\(^{WAF1}\), Mm01303209_m1 was used; other assays were custom-designed at ABL. Gene copy number assays for Dmp1, Arf, p53, grm3, and aehb1 were also performed by real-time PCR using β-actin as an internal control (26).

Western blotting

Proteins were extracted with ice-cold EBC buffer (15) with proteinase inhibitors from frozen mammary tumor cells or human breast cancer cell lines. After gel electrophoresis and transfer to nitrocellulose membranes, proteins were visualized by immunoblotting with affinity-purified polyclonal antibodies to Dmp1 (RAX; ref. 23), p53 (sc-6243G, Santa Cruz Biotech), Mdm2 (ab16896 [2A10], Abcam), p19\(^{ARF}\) (sc-32748), p14\(^{ARF}\) (ab3642, Abcam), p16\(^{Ink4a}\) (sc-1207), p21\(^{WAF1}\) (sc-1207), p21\(^{WAF1}\) (sc-397G), TBX2 (sc-17880), Pokemon (A300-548A, Bethyl, Inc.), Twist (sc-15393), or β-actin (sc-1615, sc-47778), followed by incubation of the filters with horseradish peroxidase conjugated secondary antibodies, and reaction with the enhanced chemiluminescence detection kit (Perkin-Elmer).

Cell culture, reporter assays, chromatin immunoprecipitation (ChIP), in vitro mutagenesis of the Dmp1 promoter, immunohistochemical staining, retrovirus for HER2 short hairpin RNA (shRNA), and statistical analyses are described in the Supplementary Materials and Methods.

Results

Both the Dmp1 and Arf promoters are specifically activated by HER2/neu

We tested whether murine Dmp1 and Arf promoter can be activated by overexpression of HER2 (Supplementary Fig. S1A). Both promoters were activated by HER2 expression in a dose-dependent fashion (Fig. 1A, left). Both human DMP1 and p14\(^{ARF}\) promoters were also responsive to HER2 (Supplementary Fig. S1B). The specificity of these promoter activations by HER2 was confirmed by reporter assays with p27\(^{KIP1}\), p16\(^{Ink4a}\), and Hdm2 promoters, which were all repressed by HER2 (Supplementary Fig. S1B). We then confirmed the results of reporter assays by quantitating the endogenous Dmp1 and Arf mRNA by real-time PCR in wild-type mouse embryo fibroblasts (MEF) infected with HER2 virus (Fig. 1A, middle). The Dmp1 protein induction by HER2 was confirmed by transiently expressing HER2 in 3T3 cells (Fig. 1A, right). The Dmp1 promoter activation by HER2 was not inhibited by the mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK inhibitor but was completely inhibited by the PI3K inhibitor and by the Akt inhibitors (Fig. 1B). The Dmp1 promoter was efficiently inhibited by proteosomal NF-κB inhibitor PS341 or by coexpression of a constitutively active IκB super repressor (Fig. 1B). Consistent with these findings, in vitro mutagenesis of either NF-κB site 1 or 2 significantly decreased the responsiveness of the Dmp1 promoter.
promoter to HER2 (Fig. 1C). We confirmed significant binding of the endogenous p65/relA and p52 subunits of NF-κB to the Dmp1 promoter on HER2 overexpression by ChIP (Fig. 1D, left). Binding of p65 and p52 to the Dmp1 promoter was also confirmed by tissue ChIP with lysates from MMTV-neu tumors (Fig. 1D, right).

We then mapped the HER2-responsive element on the Arf promoter. When the Dmp1/Ets site was mutated, the Arf promoter was not responsive to HER2 expression, suggesting that the promoter activation was Dmp1/Ets-dependent (Fig. 2A, middle). The Arf promoter activation by HER2 was dependent on Dmp1 because the promoter was not activated in Dmp1−/− cells (Fig. 2A, right). Binding of endogenous Dmp1 to the endogenous Arf promoter was confirmed by ChIP with lysates from four independent neu tumors (Fig. 2B). Thus, our data indicate that HER2/neu stimulates the Arf-p53 pathway through activation of the novel PI3K-Akt-NF-κB-Dmp1 signaling (Fig. 2C).

**Acceleration of neu-induced mammary tumor development in Dmp1-knockout mice**

MMTV-neu females develop multiple mammary tumors (~5 mm in diameter) with a mean latency of 7 months in FVB/N strain. Because the Dmp1 promoter is selectively activated by HER2/neu, we studied if Dmp1 and p53 proteins were induced in response to active neu in premalignant lesions (i.e., hyperplastic, nontransformed mammary glands mixed with islands of early stage tumors) isolated from 5.5-month old MMTV-neu females. Real-time PCR analysis showed upregulation of the Dmp1 mRNA in early-stage mammary tumors (Fig. 3A, left). Significant induction of the Dmp1 protein in hyperplastic premalignant lesion was confirmed by immunohistochemical staining of mammary glands from a 5.5-month-old female (Fig. 3A, third panel) as well as those adjacent to neu tumors from a 7-month-old female (Fig. 3A, fourth panel, arrows; n = 5, P < 0.001). The p53 protein was barely detectable in normal mammary glands (Fig. 3B, left),
but significant amount of p53 was induced in hyperplastic mammary glands from neu mice (Fig. 3B, middle arrow, \( P < 0.01 \)). We also observed significant induction of p21\(^{Cip1/WAF1}\) in premalignant lesions (Fig. 3B, right arrow, \( P < 0.01 \)). On the other hand, the proapoptotic p53 target Puma was not induced in early-stage neu tumors (data not shown). Our data indicate activation of the Dmp1-p53-p21\(^{Cip1/WAF1}\) signaling in premalignant hyperplastic mammary glands or early-stage mammary tumors in response to oncogenic HER2/neu signaling.

To study the cooperation between Dmp1 loss and HER2/neu overexpression/activation in vivo, we crossed MMTV-neu mice with Dmp1-null mice. Mammary glands from Dmp1\(^{-/-}\) or Dmpy\(^{-/-}\) virgin females are morphologically indistinguishable from those from wild-type females, with nearly the same staining patterns for ER, PR, and Ki67 (Supplementary Figs. S2 and S3). HER2/neu-induced mammary tumor development was significantly accelerated from 200 to 162 days in Dmp1\(^{-/-}\) and 154 days in Dmp1\(^{-/-}\) mice (\( P < 0.0001 \)), with no statistically significant differences between Dmp1\(^{-/-}\) and Dmpy\(^{-/-}\) (Fig. 3C). Analysis of genomic DNA from mammary tumors of Dmp1\(^{-/-}\) mice showed that the wild-type Dmp1 locus was retained in all the eight tumors examined (Fig. 3C). The tumors from Dmp1\(^{-/-}\) mice expressed the Dmp1 protein, showing haploid insufficiency (Fig. 3D). p53 was barely detectable in the mammary glands or tumors from Dmp1\(^{-/-}\) or Dmpy\(^{-/-}\) mice, suggesting inactivity of the p53 pathway (Fig. 3D). These data indicate that Dmp1 has a critical role both as a mediator of HER2-p53 signaling and in prevention of neu-induced mammary tumor development.

**Frequent deletion of Dmp1 in neu-induced mammary tumors**

The Dmp1 protein was often downregulated in tumor tissues compared with premalignant mammary glands in wild-type MMTV-neu mice (Fig. 3A, fourth panel, T). To investigate the molecular mechanism for this finding, we studied the gene copy numbers of Dmp1 by real-time PCR (Fig. 4A). One allele of the Dmp1 gene was deleted in 6 of 10 mammary tumors from single MMTV-neu transgenic mice and 6 of 8 tumors in double neu transgenic mice (Fig. 4A). Conversely, one allele of Arf was lost only in 1 of 10 tumors, and none of the tumors showed p53 deletion (Supplementary Fig. S4). Specific deletion of Dmp1 was further confirmed by real-time PCR analyses of grm3 and mdr1 (Fig. 4B). Hypermethylation of the Dmp1 promoter was not found in any of the randomly chosen 10 tumor DNAs from wild-type MMTV-neu mice (data not shown). The tumors with hemizygous deletion for Dmp1 (Dmp1\(^{+/HD}\)) expressed significantly lower levels of Dmp1 mRNA than those without Dmp1 deletion (Dmp1\(^{+/-}\)ND; \( P < 0.0001 \); Fig. 4C). The Dmp1 mRNA was significantly downregulated in tumors from Dmp1\(^{-/-}\) mice than in Dmp1\(^{+/-}\)ND (\( P < 0.0001 \)), but the levels were not different from those of Dmp1\(^{+/-}\)HD tumors (Fig. 4C). p19\(^{Arf}\) mRNA expression was lower in mammary tumors from Dmp1\(^{+/-}\)HD, Dmp1\(^{-/-}\), and Dmpy\(^{-/-}\) mice than in Dmp1\(^{+/-}\)ND tumors, but the levels were not significantly different among tumors from Dmp1\(^{+/-}\)HD, Dmp1\(^{-/-}\), and Dmpy\(^{-/-}\) mice (Fig. 4D). Likewise, the p53 target p21\(^{Cip1/WAF1}\) mRNA expression was significantly downregulated in mammary tumors from Dmp1\(^{+/-}\)HD, Dmp1\(^{-/-}\), and Dmpy\(^{-/-}\) mice compared with Dmp1\(^{+/-}\)ND (Supplementary Fig. S5A), whereas the expression of proapoptotic target Puma did not change significantly among the three Dmp1 genotypes (data not shown). The p19\(^{Arf}\) mRNA expression in tumors from Dmp1\(^{+/-}\)HD and Dmp1\(^{-/-}\) tumors was not significantly different from that in Dmp1\(^{-/-}\)ND, although it was downregulated in Dmp1\(^{-/-}\) (Supplementary Fig. S5B).

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**Figure 2.** Activation of the Arf promoter by HER2 and the signaling pathways that link HER2 and p53. A, activation of the Arf promoter by HER2 is dependent on Dmp1. Reporter assays were conducted in 3T3 cells (left and middle) or in Dmp1\(^{-/-}\) cells (right). The baseline (without HER2 expression vector) was set at 1.0. B, binding of endogenous Dmp1 to the Arf promoter in four different MMTV-neu tumors. C, proposed signaling pathway that links HER2/neu overexpression and p53 activation. Activation of the Dmp1 promoter is mediated by PI3K-Akt-NF-κB signaling, and induction of Arf by HER2/neu is dependent on Dmp1. This divergent hyperproliferative signaling from HER2/neu to a p53-dependent cell cycle arrest or apoptosis.

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**Figure 3.** Arf and p53 in mammary gland development and mammary tumor progression. A, activation of the Arf promoter by HER2 and the signaling pathways that link HER2 and p53. A, activation of the Arf promoter by HER2 is dependent on Dmp1. Reporter assays were conducted in 3T3 cells (left and middle) or in Dmp1\(^{-/-}\) cells (right). The baseline (without HER2 expression vector) was set at 1.0. B, binding of endogenous Dmp1 to the Arf promoter in four different MMTV-neu tumors. C, proposed signaling pathway that links HER2/neu overexpression and p53 activation. Activation of the Dmp1 promoter is mediated by PI3K-Akt-NF-κB signaling, and induction of Arf by HER2/neu is dependent on Dmp1. This divergent hyperproliferative signaling from HER2/neu to a p53-dependent cell cycle arrest or apoptosis.

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**Figure 4.** Activation of the Arf promoter by HER2 and the signaling pathways that link HER2 and p53. A, activation of the Arf promoter by HER2 is dependent on Dmp1. Reporter assays were conducted in 3T3 cells (left and middle) or in Dmp1\(^{-/-}\) cells (right). The baseline (without HER2 expression vector) was set at 1.0. B, binding of endogenous Dmp1 to the Arf promoter in four different MMTV-neu tumors. C, proposed signaling pathway that links HER2/neu overexpression and p53 activation. Activation of the Dmp1 promoter is mediated by PI3K-Akt-NF-κB signaling, and induction of Arf by HER2/neu is dependent on Dmp1. This divergent hyperproliferative signaling from HER2/neu to a p53-dependent cell cycle arrest or apoptosis.

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**Figure 5.** Activation of the Arf promoter by HER2 and the signaling pathways that link HER2 and p53. A, activation of the Arf promoter by HER2 is dependent on Dmp1. Reporter assays were conducted in 3T3 cells (left and middle) or in Dmp1\(^{-/-}\) cells (right). The baseline (without HER2 expression vector) was set at 1.0. B, binding of endogenous Dmp1 to the Arf promoter in four different MMTV-neu tumors. C, proposed signaling pathway that links HER2/neu overexpression and p53 activation. Activation of the Dmp1 promoter is mediated by PI3K-Akt-NF-κB signaling, and induction of Arf by HER2/neu is dependent on Dmp1. This divergent hyperproliferative signaling from HER2/neu to a p53-dependent cell cycle arrest or apoptosis.

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**Figure 6.** Activation of the Arf promoter by HER2 and the signaling pathways that link HER2 and p53. A, activation of the Arf promoter by HER2 is dependent on Dmp1. Reporter assays were conducted in 3T3 cells (left and middle) or in Dmp1\(^{-/-}\) cells (right). The baseline (without HER2 expression vector) was set at 1.0. B, binding of endogenous Dmp1 to the Arf promoter in four different MMTV-neu tumors. C, proposed signaling pathway that links HER2/neu overexpression and p53 activation. Activation of the Dmp1 promoter is mediated by PI3K-Akt-NF-κB signaling, and induction of Arf by HER2/neu is dependent on Dmp1. This divergent hyperproliferative signaling from HER2/neu to a p53-dependent cell cycle arrest or apoptosis.
Figure 3. Induction of Dmp1 and p53 in vivo by HER2/neu and acceleration of neu-induced mammary carcinogenesis in Dmp1-knockout mice. A, left, real-time PCR analysis of the Dmp1 mRNA in early-stage mammary tumors from MMTV-neu mice (age, 5–6 mo; tumors, 1–2 mm; n = 3). Right, detection of the Dmp1 protein in normal mammary gland (second panel), hyperplastic, nontransformed mammary glands from a MMTV-neu mouse (third panel, premalignant), and those adjacent to a MMTV-neu tumor (fourth panel, arrows). Note that once a mouse develops mammary tumor (T), the Dmp1 expression levels go down due to hemizygous gene deletion (Fig. 4A). B, left, p53 is barely detectable in normal mammary glands (arrows). Middle, induction of p53 protein in hyperproliferative mammary glands (arrow) from a MMTV-neu mouse. p53 is significantly downregulated in the tumor (T). Right, induction of the p21Cip1/WAF1 protein in premalignant lesions (arrows) from a MMTV-neu mouse. C, left, tumor-free survival of Dmp1+/+ (blue), Dmp1+/- (pink), and Dmp1−/− (green); MMTV-neu compound transgenic mice. Tumor development was significantly accelerated on Dmp1−/− and Dmp1+/- genetic backgrounds compared with wild-type mice (both P < 0.0001). Right, retention of the wild-type Dmp1 locus in mammary carcinomas from Dmp1−/−, neu mice. D, left, detection of the Dmp1 protein in Dmp1−/−, neu tumor and neighboring tissue. At the right are background signals from a mammary carcinoma from a Dmp1−/−, neu mouse. Right, p53 is barely detectable in neu-induced mammary tumors from a Dmp1−/− (left) or a Dmp1−/− mouse (right). Scale bars, 100 μm.
We then studied protein expression involved in the Arf-Mdm2-p53 tumor surveillance pathway and Ink4a/Arf modulators in neu tumors from the three Dmp1 genetic backgrounds. The Dmp1 protein expression was 2 to 10 times higher in wild-type neu tumors than in nontransgenic wild-type mammary glands from 12-week-old virgin females (Fig. 5A), reflecting the promoter activation. As expected, Dmp1<sup>ND</sup> tumors showed higher levels of Dmp1 than in Dmp1<sup>HD</sup> tumors. The Dmp1 protein expression was higher than that in normal mammary glands in some Dmp1<sup>+/−</sup>; neu tumors or at the levels of normal mammary glands in others (Fig. 5B). None of the tumors from the three Dmp1 genotypes overexpressed p19<sup>Arf</sup> or p53 at the level of the p53-mutant cell line (Fig. 5), suggesting that neu tumors of the three different Dmp1 genotypes retained wild-type p53. Sequencing of the p53 cDNAs confirmed that these tumors expressed wild-type p53 regardless of the Dmp1 genotype. Mdm2 was not overexpressed in any of the neu tumors (Fig. 5). Tbx2 overexpression was found in ∼70% of the tumors from Dmp1 wild-type mice and in 20% to 30% of Dmp1<sup>+/−</sup> and Dmp1<sup>−/−</sup> tumors (Fig. 5). Pokemon overexpression was found in nearly all the tumors from HER2/neu tumors, regardless of the Dmp1 genotype, whereas none of the mammary tumors overexpressed Twist (Fig. 5). None of the MMTV-neu tumors overexpressed Tbx3 or Bmi1 (data not shown). Together, our molecular genetic analyses of neu-induced mouse mammary

**Figure 4.** Specific deletion of Dmp1 and expression of Dmp1/Arf mRNA in neu-induced mammary tumors. A, real-time PCR analysis of the Dmp1 copy numbers in wild-type neu tumors showing Dmp1 deletion in 60% of single neu-transgenic and 75% of double neu-transgenic mice. B, the grm3 (glutamate receptor 3) gene, which is located ∼500 kb upstream from the Dmp1 locus, was not deleted in any of the neu tumors. The mdr1 (multidrug resistance 1) gene, located ∼500 kb downstream from the Dmp1 locus, was deleted in only 2 of 10 cases examined. C, relative expression of the Dmp1 mRNA in mammary carcinomas from MMTV-neu mice. Dmp1<sup>ND</sup> indicates tumor cells without Dmp1 deletion; Dmp1<sup>HD</sup> shows those with hemizygous deletion of Dmp1. D, relative expression of the Arf mRNA in mammary carcinomas from neu mice. The Arf expression was significantly downregulated in Dmp1<sup>HD</sup>, Dmp1<sup>−/−</sup>, and Dmp1<sup>+/−</sup> tumors.
tumors showed that (a) hemizygous deletion of Dmp1 is found in ~50% of wild-type neu tumors, (b) mutation/deletion/overexpression of key components of the Arf-Mdm2-p53 pathway is rare in neu tumors, (c) both p19Arf and p21Cip1/WAF1 mRNAs are significantly downregulated in Dmp1wt HD, Dmp1+/−, and Dmp1−/− tumors compared with Dmp1wt ND tumors, showing the inactivity of the p53 pathway and the mechanism of haploid insufficiency of Dmp1, (d) p16Ink4a mRNA level is low only in Dmp1−/− mammary tumors, and (e) both Tbx2 and Pokemon proteins are often overexpressed in wild-type neu tumors; Tbx2 overexpression is less frequent in Dmp1+/− or Dmp1−/− tumors, whereas Pokemon overexpression is independent of the Dmp1 genotype.

**Histopathologic features of HER2/neu tumors from Dmp1-deficient mice**

Macroscopically, tumors from Dmp1+/− or Dmp1−/− mice showed more aggressive phenotypes (i.e., high nuclear grade, local invasion, increased angiogenesis, and metastasis) than those from Dmp1+/+ mice. At sacrifice, the total tumor weight...
was significantly increased in Dmp1+/− (P = 0.039) and Dmp1+/− (P = 0.0015) mice compared with Dmp1+/- mice (Fig. 6A and B). Metastatic disease was more frequent in Dmp1+/− (4 of 26, 15.4%) or Dmp1+/- mice (4 of 37, 10.8%) than in Dmp1+/+ mice (2 of 35, 5.7%). Tumors from MMTV-neu mice were then categorized using the published grading criteria (38). Grade A is encapsulated, low-grade nodular mammary tumor with uniform nuclear size with low mitotic count (Fig. 6C, top, found in Dmp1+/+); grade B is intermediate, invasive mammary adenocarcinoma (i.e., tumor cells have broken out the lobule and begun to spread to other areas) with small and large nests of cancer cells infiltrating the mammary stroma (middle, arrow, from Dmp1+/-); grade C is a high-grade, solid, invasive carcinoma with remarkably
high mitotic figure count and central comedo necrosis with high-grade nuclei (bottom, arrow, from Dmp1+/−; ref. 38). Tumors from both Dmp1+/− and Dmp1+/+ mice showed significantly increased scores of grades B and C tumors, showing the more aggressive pattern of tumor development, although there was no significant difference in the features between samples deficient in one or two alleles of Dmp1 (Fig. 6D, top). When Dmp1 wild-type tumors were compared between HD and ND groups, tumors with Dmp1 deletion showed significantly increased scores for grade B (P = 0.0033), but not for grade C (Fig. 6D, bottom). The difference in the invasiveness between Dmp1+/HD tumors and Dmp1−/− tumors can be explained by the duration of Dmp1 deletion, which should be significantly longer in tumors from Dmp1−/− mice. Thus, our data indicate that loss of Dmp1 contributes to the more invasive and metastatic phenotypes of the more aggressive and metastatic phenotypes of mammary carcinomas in neu-transgenic mice.

Endogenous HER2 upregulates hDMP1 mRNA in human breast epithelial cells

To study whether endogenous HER2 upregulates hDMP1 in human breast epithelial cells, hDMP1 levels were quantitated by real-time PCR. We found that hDMP1 mRNA levels were significantly higher in human breast cancer cell lines with HER2 overexpression than those with low or no HER2 expression (P = 0.0076; Supplementary Fig. S6A). Downregulation of endogenous HER2 with two different shRNAs (ref. 39; >95%) resulted in significant decrease of the hDMP1 mRNA in three different HER2-amplified human breast cancer cell lines, SK-BR-3, BT-474, and HCC1569 (Supplementary Fig. S6B; HCC1569, data not shown). p14ARF mRNA also decreased in BT-474 cells treated with shRNA to HER2 (data not shown). Inhibition of PI3K, Akt, or NF-κB activity by specific inhibitors downregulated endogenous hDMP1 levels in these breast cancer cells (Supplementary Fig. S6C) and induced cell cycle arrest or apoptosis in SK-BR-3 and BT-474 cells (Supplementary Fig. S6D). Together, overexpression of HER2 increases endogenous hDMP1 through activation of the PI3K-Akt-NF-κB pathway.

Discussion

In this study, we have characterized the signaling pathway that links HER2/neu overexpression and p53 activation. Although HER2 overexpression activates both Ras-Raf-MEK-ERK and PI3K-Akt-NF-κB signaling, our study shows that HER2-Dmp1 signaling is independent of the former signaling cascade. Dependence of the Dmp1 promoter activation by NF-κB was confirmed by (a) proteasomal inhibitor PS341 treatment, (b) expression of IkBα super repressor, and (c) mutating the NF-κB sites on the Dmp1 promoter. Moreover, we confirmed the binding of endogenous p65 and p52 subunits of NF-κB to the endogenous Dmp1 promoter in HER2 virus–infected cells as well as in mammary tumors from MMTV-neu mice by tissue ChIP. It has been reported that phosphorylation of p65 Ser505 in transactivation domain 1 by IKKα/β/ε or other kinases can stimulate p65 transactivation (40). Conversely, phosphorylation of p65 Thr505 in transactivation domain 2 by Chk1 results in transcriptional repression of some NF-κB target genes by increased association of p65 with histone deacetylase inhibitor 1 (41, 42). Phosphorylation of p65 at Thr505 occurs when the cells are exposed to genotoxic stimuli. Thus, NF-κB plays roles in both activation (HER2/neu; this study) and repression (genotoxic stimuli; ref. 24) of the Dmp1 promoter dependent on the stress the cells receive.

It was reported that p19ARF inhibits HER2/neu-mediated oncogenic growth by antagonizing Akt-mediated p27kip1 phosphorylation and increasing p21kip1 stability (43). Our study showed that the Arf promoter is activated by HER2/neu. Wild-type MMTV-neu tumors that retained two alleles of Dmp1 expressed the Arf mRNA at levels 2 to 40 times higher than that in normal mammary epithelial cells. Induction of p19ARF by HER2/neu is largely dependent on Dmp1 because (a) the Arf promoter activation was not found in Dmp1-deficient cells and (b) Arf mRNA levels were significantly lower in Dmp1−/−, Dmp1−/−, and Dmp1+/HD; neu tumors than in Dmp1+/−ND. The critical role of the Dmp1-Arf-p53 pathway in preventing mammary tumorigenesis was shown by immunohistochemical staining of preneoplastic regions found in early-stage neu tumors, where we found significant upregulation of Dmp1, p53, and p21kip1/WAF1 proteins. Although significant induction of Arf mRNA was detectable by real-time PCR in Dmp1+/−ND; neu tumors, immunohistochemical demonstration of p19ARF in premalignant mammary tissue was technically difficult possibly because the absolute expression levels of p19ARF were very low. It is generally believed that very low levels of Arf are enough to show tumor-suppressive activity and that further induction provides the selective pressure for the emergence of tumors that have inactivated the gene (44).

Interestingly, the mouse Dmp1 gene was hemizygously deleted in ~50% of neu mammary tumors with significant downregulation of the Dmp1 protein. The gene deletion was limited to the Dmp1 locus in 80% of the mouse tumors, according to our analysis of neighbor gene deletions by real-time PCR. However, neu-induced mammary tumors are different from human breast cancers in that the Ink4a/Arf or p53 locus is not frequently involved. Mdm2 overexpression was not observed in any of the neu tumors, regardless of the Dmp1 genotype. In contrast, we found frequent overexpression of Ink4a/Arf repressors, Tbx2 and Pokemon, in HER2/neu tumors. The frequency of Pokemon overexpression did not change significantly in Dmp1−/− and Dmp1−/− tumors, whereas the frequency of Tbx2 overexpression was decreased from 70% to 20% to 30% in Dmp1−/− and Dmp1−/− tumors. This indicates that Dmp1 deletion may alleviate the function of Tbx2 overexpression to some extent. It has been reported that Tbx2 is amplified in 8.6% to 21.6% of sporadic human breast carcinomas (45), and ectopic expression of Tbx2 results in DNA polyploidy and cisplatin resistance (46). On the other hand, very little is known about the role of Pokemon in human breast cancer (47). Further studies will be required to reveal how these Ink4a/Arf repressors collaborate with Dmp1 loss in breast (or mammary) carcinoma development.
Our current study clearly shows the haploid insufficiency of Dmp1 in neu-induced mammary tumor suppression. Consistent with these findings, the p19Arf and p21Cip1/WAF1 mRNA levels were significantly downregulated in both Dmp1+/− and Dmp1−/− tumors, with no differences between the two cohorts. Of note, downregulation of Arf and p21Cip1/WAF1 was also observed in Dmp1−/− cells, indicating that naturally occurring hemizygous deletion of Dmp1 inactivates the Arf-p53 pathway as well. In either case, loss of Dmp1 was associated with more aggressive disease than Dmp1+/− tumors. In contrast, p16Ink4a mRNA was downregulated only in mammary tumors from Dmp1−/− mice compared with Dmp1+/− mice, suggesting differential regulation of the Ink4a and Arf promoters by Dmp1. This can be explained by the fact that the p16Ink4a promoter lacks typical Dmp1 consensus sequences, at least within 500 bp from the transcription initiation site (17, 48).

In conclusion, our study shows a novel signaling cascade that links HER2/neu and p53. Because Dmp1 is induced in premalignant tissues, activation of Dmp1 by small molecules may be a reasonable approach to prevent breast cancer development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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