Mutant p53 Amplifies Epidermal Growth Factor Receptor Family Signaling to Promote Mammary Tumorigenesis

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Abstract

The EGFR family (ErbB2/Her2 and EGFR/ErbB1/Her1) often modulates the transcriptional program involved in promoting mammary tumorigenesis. In humans, the majority of ErbB2-positive sporadic breast cancers harbor p53 mutations, which correlate with poor prognosis. Also, the extremely high incidence of ErbB2-positive breast cancer in women with p53 germline mutations (Li–Fraumeni syndrome) suggests a key role of mutant p53 specifically in ErbB2-mediated mammary tumorigenesis. To examine the role of mutant p53 during ErbB2-mediated mammary tumorigenesis, a mutant p53 allele (R172H) was introduced into the (MMTV)-ErbB2/Neu mouse model system. Interestingly, we show in heterozygous p53 mice that mutant p53 R172H is a more potent activator of ErbB2-mediated mammary tumorigenesis than simple loss of p53. The more aggressive disease in mutant p53 animals was reflected by earlier tumor onset, increased mammary tumor multiplicity, and shorter survival. These in vivo and in vitro data provide mechanistic evidence that mutant p53 amplifies ErbB2 and EGFR signaling to promote the expansion of mammary stem cells and induce cell proliferation.

Implications: This study identifies mutant p53 as an essential player in ErbB2 and EGFR-mediated mammary tumorigenesis and indicates the potential translational importance of targeting mutant p53 in this subset of patients with breast cancer. Mol Cancer Res; 13(4); 1–12. ©2015 AACR.

Introduction

Breast cancer in women is the most frequent life-threatening disease, which has a complex, heterogeneous genetic, and biochemical background. No single genomic alteration can be regarded as decisive for its formation and progression. However, a few key players have been identified and among them are the p53 tumor suppressor and members of the EGFR family, such as Her2 (human EGF receptor 2, alias ErbB2) and EGFR (alias ErbB1/Her1).

According to a recent comprehensive analysis, mutations in the p53 gene are the most frequent genetic alteration found in breast cancer (1), with high occurrence in the Her2-positive (72%) and basal-like (80%) breast cancer subtypes. However, mutations in the p53 gene are rather rare in Luminal A (12%) and Luminal B (29%) breast cancers, suggesting that mutp53 may cooperate with certain oncogenic pathways to promote mammary tumorigenesis. The significance of mutant p53 (mutp53) in Her2-positive breast cancer initiation is also supported by the frequent occurrence of breast cancer in Li–Fraumeni syndrome (LFS), a hereditary cancer disorder associated with p53 germline mutations. Breast cancer is the most common event in LFS, accounting for 49% of LFS women (2). Importantly, patients with LFS with germline p53 mutations have more than 70% incidence of Her2 breast cancer compared with the 20% to 30% of sporadic breast cancer with deregulated Her2 (2, 3). Therefore, mutp53 germline mutations predispose LFS women for Her2-positive breast cancer development, suggesting a critical role of mutp53 in pathogenesis specifically of this subtype of breast cancer. In addition, mutp53 Her2 breast cancers have a worse prognosis and susceptibility to metastatic recurrence than wild-type p53 (wtp53) Her2 breast cancer (4, 5). Therefore, mutp53 and Her2 oncogenes appear to strongly cooperate. While indicating oncogenic synergy between mutp53 and Her2 in the clinic, the cellular and molecular basis of this cooperation is completely unknown.

A rising interest in cancer stem cells (SC), combined with the idea that SCs and/or early progenitors might be targets of neoplastic transformation (6, 7), drew attention to the role of p53 and its tumor suppression activities in SC homeostasis. Recent findings suggest the intriguing possibility that wtp53 carries out its tumor suppressor function by inhibition of SC symmetric division and blocking of reprogramming of somatic/progenitor cells into SCs (8). The observation that p53 controls SC maintenance is coupled with the notion that p53 mutations may contribute to SC evolution. Compared with “simple” p53 deficiency, the presence of mutp53 markedly enhances the efficiency of reprogramming somatic fibroblasts into induced pluripotent SCs (iPS; ref. 9). This suggests that reprogramming of somatic mutp53 cells may result in the generation of stem-like cells with malignant potential. Also, we (10) and others (11) have shown that mutp53 promotes expansion of normal progenitors of different tissue origins, compared with the p53 null allele in vivo. Yet, the underlying mechanisms of these observations remain to be elucidated.
ErbB2/Her2 is a tyrosine kinase transmembrane receptor that forms heterodimers with other EGFR family members, including EGFR/Her1, to stimulate oncogenic signaling (12). Overexpression of ErbB2 in breast cancer activates pathways that promote cell proliferation, reduce apoptosis, and increase metastasis (13). Intriguingly, recent reports demonstrated the novel oncogenic proliferation, reduce apoptosis, and increase metastasis (13).

Here, we demonstrate that mutp53 cooperates with oncogenic ErbB2 signaling in mammary tumor development using a newly generated mammary tumor virus (MMTV)-ErbB2/Neu mouse model containing a knock-in mutp53 R172H allele. In heterogeneous animals, containing one wt p53 allele, the mutp53 allele accelerates ErbB2-mediated mammary tumorigenesis compared with their null p53 allele counterparts. In the ErbB2 mouse model, the mutp53 allele induces multicentric mammary tumor formation, earlier tumor onset, and shorter survival, which may be mediated by the expansion of normal mammary progenitors and/or cancer SCs. Moreover, we elucidated a mechanistic underpinning of mutp53-EGFR (EGFR/ErbB2) cooperation, which we propose is based on novel tumorigenic activity of mutp53. This study for the first time provides evidence that mutp53, via augmented ErbB2/EGFR signaling, promotes the proliferation of mammary cells and expands mammary SC populations. This establishes a new role of mutp53 in breast cancer stem cell biology and opens opportunities for targeting mutp53 as a therapeutic strategy in Her2/EGFR-positive breast cancer.

Materials and Methods

Cells

Human mutp53 breast cancer cells MDA231 (p53R280K) and MDA231 cell line was previously described (18). MDA231 stably overexpressing native ectopic p53 (R280K) or vector only was generated by Lipofectamine (Invitrogen) transfection followed by selection in G418. All cells were cultured in 10% FCS/DMEM.

RNAi

Pools of four different siRNA duplexes specific for p53R280K or scrambled controls were transfected with Lipofectamine (RNAITM/RNAiMAX, Invitrogen). Cells were harvested 48 hours later and analyzed.

Immunoblot analyses and immunoprecipitations

For immunoblot analyses, equal total protein of cell lysates (2.5–20 µg) were detected with antibodies to mouse p53 (FL393), human p53 (Pab 1801), and aldehyde dehydrogenase (ALDH) 1/2 (all Santa Cruz Biotechnology), AKT, pAKT, Erk, pErk, EGFR, EGRF-Tyr845P (all Cell Signaling Technology), ErbB2, and Actin (all Neomarkers).

Mammary epithelial cell cultures

Mammary glands were dissected from 8-week-old virgin female mice and sequentially digested at 37°C for 2 hours in collagenase/hyaluronidase, 0.05% Trypsin, DNase I, and Dispase (Stem Cell Technology). The ensuing cell suspension was treated with red blood cell lysis buffer, rinsed with PBS, and passed through a 40-µm mesh after resuspension in Opti-Mem medium (Gibco). Mammary epithelial cells (MEC) were plated on gelatin-coated plates and grown in CMF-BM1 medium (Cell-N-Tec).

Dissociation of normal mammary tissue and mammosphere assays

For mammosphere experiments, single cell suspensions of MECS or MDA231 cells were plated on ultralow attachment 6-well plates at a density of 20 × 10^3 cells/well for 10 days. Subsequent cultures after dissociation of primary spheres were replated. Mammosphere cultures were grown in a serum-free mammary epithelium basal medium as previously described (19).

Mice

MMTV-ErbB2 mice harboring activated ErbB2 [strain FVBN-Tg (MMTV-ErbB2)NK1Mul/J] were from Jackson Laboratories. p53 R172H (called p53R172H) and control p53 null (p53+/−) mice (C57Bl6/J background) were a gift from G. Lozano (University of Texas, MD Anderson Cancer Center) (20). p53 mice were interbred to generate p53R280K−/− mice. Compound p53R280K−/−;ErbB2 mice were generated by crossing ErbB2 into the p53−/− background and then breeding the p53−/−;ErbB2 progeny with p53R280K−/− animals. p53R280K−/−;ErbB2 were then crossed with p53−/− animals to generate p53R280K−/−;ErbB2 and p53−/−;ErbB2 females for analysis. These F2 mice were of a mixed C57Bl6/J/FVB/N background, p53−/−;ErbB2 versus p53−/−;ErbB2 animals were generated by crossing a p53 R172H−/− male on a pure C57Bl6/J background with a MMTV-ErbB2 female on a pure FVB/N background, generating 50% FVB/N and 50% C57Bl6/J genetic background littermates. Littermates were used for all analyses. Mice were regularly monitored and euthanized when they became moribund or the largest tumor reached 4 cm³. Careful necropsies were performed and tumors and all major organs collected, fixed in 10% formalin, embedded in paraffin, and sectioned for histopathologic analysis. For survival analysis, P values were determined by log-rank analysis using SigmaPlot software. All animal studies were approved by the institutional animal care and use committee at Stony Brook University (Stony Brook, NY).

Aldefluor assay and flow cytometry

To measure ALDH activity, the Aldefluor assay was performed according to the manufacturer’s (Stemcell Technologies) protocol. Dissociated single cells were suspended in Aldefluor assay buffer containing the ALDH substrate, Bodipy-aminoacetaldehyde (BAAA) at 1.5 mmol/L, and incubated for 45 minutes at 37°C. To distinguish between ALDH-positive and ALDH-negative cells, a fraction of cells were incubated under identical conditions in the presence of a 10-fold molar excess of the ALDH inhibitor, diethylaminobenzaldehyde (DEAB). The ALDH-positive population of cells was measured by FACS according to manufacturer’s guidelines.

IHC analysis

IHC analysis was conducted as previously described (21). Protein expression was analyzed using rabbit p53 (CM5) antibodies (Vector Laboratories) at 1:1,000 overnight, and visualized using ABC and DAB kits from (Vector Laboratories). Slides were counterstained with hematoxylin.
Tissue microarrays

Tissue arrays of breast invasive ductal carcinomas (75 cases/150 cores; BR1504, Biomax) with known TNN4, pathologic grade, and markers (ER, PR, HER2, and Ki-67) were stained with antibodies to p53 (DO1, Santa Cruz Biotechnology). Correlation between p53 positivity and percentage of Ki-67-positive cells was determined by the t test and the Mann–Whitney test.

Results

Mutant R172H p53 promotes mammary tumorigenesis in the ErbB2 mouse model

Although ample clinical evidence suggests strong oncogenic cooperation between mutp53 and ErbB2/Her2 in human breast cancer, its underlying mechanism remains unknown. To date, the best available LFS mouse models are ‘straight’ mutp53 knockin mice (20, 22). However, in contrast with human LFS patients, which mainly develop Her2-positive breast cancer in women, these mice mainly develop hematopoietic malignancies and are not suitable for breast cancer research (20, 22). The extremely high incidence of Her2-positive breast cancer in LFS women (2, 3) suggests the essential role of mutp53 in specifically Her2-mediated mammary tumorigenesis. To faithfully recapitulate LFS disease and explore oncogenic activity of mutp53 in Her2-positive breast cancer, we generated a novel mutp53 mouse model by crossing the hotspot mutp53 knockin allele p53R172H (referred to as p53H hereafter) into the classic MMTV-ErbB2 Tg mouse (23). Four experimental cohorts, all expressing a single copy of activated ErbB2, were generated: homozygous p53H+/H;ErbB2 and p53−/−;ErbB2 littermates, and heterozygous p53H+/−;ErbB2 and p53−/+;ErbB2 littersmates.

Compared with heterozygous animals, homozygous mice (p53H+/H;ErbB2 and p53−/−;ErbB2) show accelerated tumorigenesis, due to rapid development of aggressive mammary tumors and lymphomas. Fifty percent of p53−/−;ErbB2 and 37% of p53H+/H;ErbB2 mice developed mammary tumors only, whereas 43% of p53−/−;ErbB2 and 52% of p53H+/H;ErbB2 mice developed lymphomas concurrently with mammary tumors or only lymphomas (Table 1). The survival curves of p53−/−;ErbB2 and of p53H+/H;ErbB2 mice were identical (median age of death 135.5 and 137 days, respectively), seemingly due to the aggressive nature of ErbB2-mediated disease and high incidence of lymphoma (Fig. 1A). In contrast, there is a marked difference in both tumor-free survival and overall survival (Fig. 1A) between the heterozygous cohorts of p53H+/−;ErbB2 and p53−/−;ErbB2 mice (median age of death 278 and 329 days, respectively). Heterozygous cohorts developed mainly mammary tumors (86% of p53−/−;ErbB2 and 95% of p53H+/−;ErbB2 mice; Table 2). A more aggressive phenotype caused the shorter survival of p53H+/−;ErbB2 mice, reflected by development of twice the number of simultaneously initiated, multicentric tumors that arise in single or multiple mammary glands in p53H+/−;ErbB2 females (average 6.1 tumors per mouse) compared with p53−/−;ErbB2 mice (average 2.7 tumors per mouse; Fig. 1B; Table 2). Strikingly, some p53H+/−;ErbB2 animals developed up to 15 visible individual mammary tumors. Homozygous p53H+/H;ErbB2 mice, which contained fewer mammary tumors compared with p53H+/−;ErbB2 mice, also developed twice more mammary tumors (2.5 tumors/mouse) compared with p53−/−;ErbB2 mice (1.5 tumors/mouse). Apparently, the short lifespan of homozygous animals did not provide sufficient time to observe the drastic differences in both survival and tumor multiplicity between p535 homozygous genotypes.

An important feature of mutp53 cancers is the tumor-specific stabilization of mutp53 proteins, which is thought to be critical for oncogenic activity of mutp53 (24–26). Because the mechanisms and physiologic consequences of mutp53 stabilization in cancer may have significant translational impact, we evaluated the levels of p53 in vivo in the mutp53/ErbB2 mouse model. Mutp53, but not wt p53, is stabilized in mammary tumors of both homozygous and heterozygous mice (Fig. 1C). Mutp53 staining in mammary tumors of p53H+/H;ErbB2 mice was heterogeneous with less than 50% of the tumor cells showing prominent nuclear mutp53 staining (Fig. 1C). However, the majority of primary mammary tumors from p53H+/H;ErbB2 animals have strong nuclear mutp53 expression (Fig. 1C). To determine whether wt p53 activity in p53H+/H;ErbB2 animals prevents mutp53 stabilization, tumors from an additional cohort of p53H+/−;ErbB2 mice were analyzed. p53 nuclear accumulation in p53H+/−;ErbB2 tumors, on average, was higher than in p53H+/+;ErbB2 tumors but lower than in p53H+/−;ErbB2 tumors (Fig. 1C). These results imply that even in the presence of a mutp53 allele, the remaining wt p53 allele preserves partial transcriptional activity and may to some extent promote mutp53 degradation via MDM2 induction (26). Nevertheless, elevated mutp53 protein levels in mammary tumors of p53H+/−;ErbB2 mice appear to contribute to more aggressive disease compared with p53−/−;ErbB2 mice in the context of oncogenic ErbB2 stimulation (Fig. 1A).

Mutant p53 expands the population of cells with stem-like properties

Stabilization of mutp53 in mammary tumors and increased mammary tumor multiplicity in the p53H+/−;ErbB2 animals suggests a novel tumor promoting function of mutp53 in ErbB2-driven mammary cancer initiation. Tumor initiation is thought to be attributed to the presence of a specific population of cells, called tumor-initiating cells (TIC) with stem cell properties (7). Therefore, the observed phenotype in the mutp53/ErbB2 model may be a physiologic consequence of an expansion of mammary SCs and/or progenitors. Thus, we hypothesize that mutp53 promotes the acquisition of stem-like properties within mammary cell populations and confers a physiologic expansion of SCs and/or progenitors in breast tissue.

We first tested whether mutp53 presence alone is sufficient to expand mammary luminal progenitors similar to what was previously described for hematopoietic and mesenchymal progenitors (10). The significance of luminal progenitors in initiation of Her2-positive breast cancer has been shown in a number of recent studies. First, Lo and colleagues analyzed numerous putative stem/progenitor markers and identified luminal progenitors as a main mammary cell population that gives rise to Her2-induced

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**Table 1. Tumor spectrum and tumor multiplicity in p53−/−;ErbB2 and p53H+/−;ErbB2 mice**

<table>
<thead>
<tr>
<th>Tumor types</th>
<th>p53−/−;ErbB2 (n = 16)</th>
<th>p53H+/−;ErbB2 (n = 19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary adenocarcinoma</td>
<td>8 (50%)</td>
<td>7 (37%)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1 (6.25%)</td>
<td>5 (26%)</td>
</tr>
<tr>
<td>Mammary + lymphoma</td>
<td>6 (37.5%)</td>
<td>5 (26%)</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>1 (6.25%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>Average mammary tumor</td>
<td>1.55</td>
<td>2.5</td>
</tr>
<tr>
<td>number per mouse</td>
<td></td>
<td></td>
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</tbody>
</table>
TICs (27). Second, gene profiling of MMTV-Her2/Neu murine mammary tumors largely overlaps with the gene signature of luminal progenitors, suggesting that these tumors can arise from the mammary luminal progenitor pool (28). Finally, PI-MECs (parity induced mammary epithelial cells), which are thought to be a cancer cell of origin in Her2/Neu-driven tumorigenesis, are part of the luminal progenitor pool of normal mammary ducts (29).

To examine the role of mutp53 in the expansion of the luminal progenitor pool, we chose to isolate MECs from homozygous p53H/H and p53H/C0 but not heterozygous females. As previously shown by Cicalese and colleagues (8), wt p53 reduces symmetric

Table 2. Tumor spectrum and tumor multiplicity in p53H/+.ErbB2 and p53H+/ErbB2 mice

<table>
<thead>
<tr>
<th>Tumor types</th>
<th>Virgin (n = 19)</th>
<th>Parous (n = 22)</th>
<th>Virgin (n = 18)</th>
<th>Parous (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammary adenocarcinoma</td>
<td>12 (86%)</td>
<td>13 (95%)</td>
<td>17 (94%)</td>
<td>17 (94%)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1 (7%)</td>
<td>1 (7%)</td>
<td>0</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>2 (14%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Non mammary carcinoma</td>
<td>0</td>
<td>1 (7%)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Average mammary tumor number per mouse</td>
<td>2.7</td>
<td>6.1</td>
<td>6.5</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Figure 1. Mutant R172H p53 promotes mammary tumorigenesis in the ErbB2 mouse model. A, mutant p53 R172H allele (p53H) promotes earlier mammary tumors onset and death in ErbB2 mouse model. Kaplan–Meier tumor-free survival curves of p53H+/ErbB2 (red curve, n = 22) versus p53−/−;ErbB2 (black curve, n = 22; solid lines) mice and p53H+/ErbB2 (red curve, n = 11) versus p53−/−;ErbB2 (black curve, n = 12) mice (dashed lines). P values are indicated. For survival curves, only mice developed mammary tumors were selected. B, left, mutant p53 R172H allele promotes tumor multiplicity. Average number (±SD) of mammary tumors per mouse per genotype. Right, representative images of p53H+/ErbB2 versus p53H−/− ErbB2 mice. Arrows indicate individual mammary tumors. C, p53 levels in mammary tumors from animals with indicated genotypes were examined by IHC. Paraffin-embedded mammary tumors were stained with a p53 (CM5) antibody. Representative images (×10).
Mutant p53 Amplifies ErbB2 and EGFR

Figure 2.

Mutant p53 expands the population of cells with stem-like properties. A, mutant p53 R172H promotes the expansion of luminal progenitors in primary mammary epithelial stem cells in ErbB2 mice. Size comparison of mammospheres were generated from primary MECs isolated from normal mammary glands of p53H/H;ErbB2 and p53H/H mice compared with p53 null littermates. Parous p53–/+;ErbB2 parous littermates (6.5 tumors per mouse) compared with p53–/–;ErbB2 parous littermates (6.5 tumors per mouse). E, mutant p53 is highly stabilized in lung metastasis compared with heterogenous p53 staining in primary tumors from the same mouse. Representative image. Matching metastatic lungs/tumor specimen derived from 5 independent mice were analyzed. p53 IHC, x10 magnification. F, mutant p53 allele changes cell morphology from epithelial in p53–/–;ErbB2 MECs to mesenchymal in p53H/H;ErbB2 MECs. x4 magnification.

The mammary gland is a highly dynamic tissue, undergoing significant morphologic changes during puberty, pregnancy, lactation, and involution. It is composed of two major epithelial division and replicative potential of mammary SCs by regulating the polarity of cell division. Therefore, presence of the wt p53 allele would complicate interpretation of the role of mutp53 in expansion of mammary progenitors in heterozygous MECs. Thus, FACS cell sorting was used to analyze the number of luminal progenitors (CD61+CD24highCD29low; ref. 27) derived from normal mammary of 8-week-old mutant p53H/H mice in comparison with p53–/– littermates and wt p53 controls. The luminal progenitor pool was significantly expanded in p53H/H mice, independent of the presence of the activated ErbB2 oncogene, compared with p53–/– and wt p53 animals (Fig. 2A). This suggests that mutp53 alone can drive the expansion of mammary progenitors and possibly SC populations. Mutp53 also induces stem-like properties of immortalized mouse MECs compared with p53–/– MECs as shown by increased expression of ALDH (Fig. 2B), which identifies MECs with SC characteristics (30). Interestingly, the highest level of ALDH was detected in MECs expressing both mutp53 and ErbB2 (Fig. 2B), which confirms the synergistic oncogenic cooperation of mutp53 and ErbB2 in the expansion of a cell population with stem-like properties.

Previous studies demonstrated that the number of mammospheres generated upon serial passaging provides an indirect measure of mammary SC self-renewal capacity, while mammosphere size reflects progenitor cell proliferation (19). Therefore, mammospheres were generated from primary MECs isolated from normal mammary glands of p53H/H;ErbB2 and p53–/–;ErbB2 littermates. p53H/H;ErbB2 MECs consistently produced a greater number and larger mammospheres (2-fold increase in average size) compared with p53–/–;ErbB2 MECs (Fig. 2C). This provides evidence that mutp53 induces mammary SC presence and proliferation in ErbB2 animals.

The mammary gland is a highly dynamic tissue, undergoing significant morphologic changes during puberty, pregnancy, lactation, and involution. It is composed of two major epithelial
populations, myoepithelial and luminal cells, which derive from myoepithelial and luminal progenitors, respectively (31). A third and intriguing population of mammary progenitors, parity-induced MECs (PI-MECs), are pregnancy-responsive, apoptosis-resistant (in involution), self-renewing progenitors that undergo immense proliferation during pregnancy to give rise to milk-producing alveoli (6, 32). A percentage of PI-MECs survive post-lactational involution after each round of pregnancy, which results in an accumulation of these progenitors in nonparous mammary glands compared with virgins (32). The surviving PI-MECs self-renew and give rise to ductal and alveolar structures (6, 33). It was previously reported that PI-MEC progenitors are targets of ErbB2-mediated tumor transformation and animals garner an increased susceptibility to ErbB2-mediated tumorigenesis as the number of PI-MEC progenitors increase with each pregnancy (29). In agreement with this observation, both parous p53H/H;ErbB2 and p53−/−;ErbB2 have accelerated tumorigenesis compared with their virgin counterparts (Table 2 and Fig. 2D). Parous mice of both genotypes developed primarily mammary tumors and had earlier tumor onset and shorter survival compared with virgins (data not shown). Nevertheless, parous p53H/H;ErbB2 mice show the higher number of mammary tumors (10.3 tumors per mouse) compared with p53−/−;ErbB2 parous littermates (6.5 tumors per mouse Fig. 2D). These data could imply that mutp53 may target and promote the expansion of the PI-MEC progenitor pool, similar to its effect on other tissue progenitors (10, 11). However, more rigorous studies are required to definitively address this question.

It is well established that primary and secondary tumor initiation sites, and the acquisition of epithelial–mesenchymal transition (EMT) features, are attributed to the presence of SCs (7, 34). Although p53 staining of primary mammary tumors from p53H/H;ErbB2 mice was heterogeneous (Fig. 1C), secondary metastatic lesions in the lungs of the same mice had homogeneous and highly stabilized mutp53 (Fig. 2E). In contrast, there was no stabilization of wtp53 in metastases in p53−/−;ErbB2 littermates (data not shown). In accordance, we found that the presence of the mutp53 allele changes MEC morphology from epithelial in p53−/−;ErbB2 to mesenchymal in p53H/H;ErbB2 (Fig. 2F). This suggests that primary tumor cells with highly stabilized mutp53 stimulate invasion have higher survival advantage and/or tumor-initiating potential at the metastatic site, which might be a physiologic consequence of mammary SC expansion in the presence of the mutp53 allele.

In sum, our data suggest a shift to a more aggressive mammary tumor phenotype when the mutp53 allele is present in ErbB2 transformed mammary cells of both virgin and parous animals, which, at least in part, is mediated by the expansion of mammary progenitors and/or SCs.

Mutant p53 promotes mammary cells proliferation

The other tumor-promoting function of mutp53 in ErbB2 context, which may not relate to SC biology, is a stimulation of mammary cell proliferation. Early stages of breast cancer (hyperplasia and ductal carcinoma in situ) are characterized by an increased proliferation of epithelial cells, a loss of acinar organization, and filling of the luminal space, followed by acquisition of invasive behavior at the later stages of mammary tumorigenesis. Activation of ErbB2 in acini induces the proliferation and generation of multilocular structures with filled lumen, indicating that ErbB2 activation overcomes the growth inhibitory effect of polarized epithelial cells (35). Therefore, mutp53-mediated amplification of ErbB2 signaling may lead to enhanced proliferation of mammary luminal cells and thereby promote tumor initiation. Growth curve analysis of primary MECs isolated from p53H/H;Erbb2 and p53−/−;Erbb2 mammary glands confirmed that the presence of the mutp53 allele promotes more rapid proliferation compared with the p53 null (Fig. 3A). Our data imply that mutp53 provides MECs a growth advantage allowing cells to bypass contact inhibition to greater extent than p53 null MECs. These hyperproliferative properties of p53H/H;Erbb2 MECs may closely relate to disruption of mammary tissue architecture, characteristic for early-stage mammary tumorigenesis in vivo.

To test whether mutp53-enhanced proliferation holds true in human mammary tumors, we examined tissue arrays of 150 breast cancer biopsies with known molecular status (ER+, PR−, Her2+). These hyperproliferative properties of p53H/H;Erbb2 MECs may closely relate to disruption of mammary tissue architecture, characteristic for early-stage mammary tumorigenesis in vivo.

Figure 3. Mutant p53 promotes mammary cell proliferation. A, to measure cell growth rates, MECs of different genotypes were plated at day 0 and counted at indicated time points. Representative graph from two independent experiments. B, mutant p53 enhances cells proliferation in Her2-positive human cancers. Tissue microarrays of 150 breast cancers biopsies with known molecular status (ER+, PR−, Her2+, or triple negative) and percentage of Ki-67-positive cells in every individual core. p53 staining was used as surrogate marker for p53 mutation. p53 staining correlates with Ki-67 positivity in Her2-positive breast cancer biopsies. (P = 0.01 t test, P = 0.001 Mann–Whitney test).
Mutant p53 allele amplifies ErbB2 signaling in vivo

To understand how mutp53 may enhance SC behavior in the ErbB2 context, we examined the pathways that are known to be involved in acquisition of stem-like properties in primary mammary tumors originating from p53H/+;ErbB2 and p53−/−;ErbB2 mice. To this end, we examined 68 primary tumors originating in virgin p53H/+;ErbB2 and p53−/−;ErbB2 mice. We did not observe statistically significant differences in Notch, Sox9, Slug, and Oct4 (7, 34, 36) by immunoblot and qRT-PCR analyses (data not shown), which can be explained by the low frequencies of the SCs/progenitors in a tumor “bulk” (8). However, we found that both total ErbB2 ($P = 0.0066$) and activated p-ErbB2 ($P = 0.0005$) levels were elevated in the presence of the mutp53 allele (Fig. 4A and B). Concurrently, there was more activated forms of p-ERK, downstream effectors of ErbB2 activation (13), in p53H/+;ErbB2 tumors compared with p53−/−;ErbB2 tumors (Fig. 4A). This suggests mutp53 amplifies ErbB2 signaling in vivo.

Previous studies provided strong evidence that ErbB2-mediated tumorigenesis and invasion might be determined by its effects on normal and malignant mammary stem/progenitor cells (14–16). Consistent with our current in vivo data, we recently showed in vitro that RNAi-mediated downregulation of mutp53 in ErbB2-positive SKBR3 human breast cancer cells promotes ErbB2 degradation (37). Conversely, ectopic overexpression of the R175H p53 mutant increases ErbB2 levels in SKBR3 cells (37). Hence, mutp53, via amplification of ErbB2 signaling, may promote the expansion of the SC pool, leading to increased metastatic recurrence, as observed in mutp53/HER2-positive patients with breast cancer (4).

Mutant p53 modulates breast cancer stem cells via amplification of EGFR signaling

Independent of mutp53 synergizing with ErbB2 signaling, our data also indicate that mutp53 can increase the luminal progenitor pool in the absence of ErbB2 oncogenic stimulation (Fig. 2A). This suggests that other pathways, besides ErbB2, may be involved in the induction of stem-like properties of mammary cells in a mutp53-dependent manner. EGFR (ErbB1/Her1) is another member of the FGFR family that is closely related to ErbB2 (12). Sustained activation of EGFR can play an important role for high self-renewal potential, survival, invasion, and metastases of cancer stem/progenitor cells and their progenies (17). Previous data, from others and us, revealed that mutp53 can enhance EGFR signaling by increasing its recycling efficiency (38) and/or stability (37).

To test whether mutp53 expands a population of cells with stem-like properties via modulation of EGFR signaling, we used the EGFR-positive human breast cancer cell line MDA231, which expresses the p53 R280K-mutant protein. We first generated a stable MDA231 cell line that ectopically overexpressed native mutp53 (R280K), referred to as MDA231-R280K (Fig. 5A). The elevated level of mutp53 increased the size and number of mammospheres, which are key characteristics of self-renewal ability of SCs. Moreover, the replicative potential of mammospheres derived from MDA231-R280K cells increased progressively during passaging (Fig. 5A, right) indicating an enrichment of the cancer SC pool. Mutp53 overexpression in MDA231 cells also increased the Aldefluor-positive/ALDH population compared with vector transfected cells, indicating that mutp53 can enhance the presence of SCs in MDA231 cells (Fig. 5B).

An important question is whether mutp53 could be a relevant target for suppression of breast cancer stem cells. Previous data from our laboratory and others have shown that acute RNAi or pharmacologic downregulation of mutp53 inhibits the invasive properties of human MDA231 breast cancer cells and tumorigenicity in xenograft assays (18, 39). However, whether this effect is related to self-renewal properties of breast cancer cells has not...
been evaluated. We generated MDA231 cells stably expressing tetracycline inducible shp53 to determine the effect of mutp53 expression on the cancer SC population. MDA231 cells containing tet-inducible shp53 were cultured in conditions that allow mammosphere formation and Western blot analysis demonstrated the efficiency of mutp53 downregulation upon introduction of tetracycline (Fig. 5C, bottom). Serial mammosphere replating of mock-treated cells had a significant pool of cells with self-renewal properties, while mutp53 knockdown dramatically decreased mammosphere formation (Fig. 5C, top). Consistent with the mammosphere assay, control shRNA-transfected cells contained a 12% Aldefluor-positive population, which is indicative of SCs, whereas mutp53 knockdown reduced this population by 2-fold (Fig. 5D). These results provide direct evidence that mutp53 regulates the self-renewal of breast cancer stem cells.

Importantly, we demonstrated that mutp53 levels are a critical regulator of EGFR signaling by using these mutp53 overexpressing and knockdown lines. In MDA231 cells, total and phospho-activated EGFR levels were increased in the presence of overexpressed mutp53 and decreased after RNAi-mediated ablation of mutp53 independent of exposure to ectopic EGF (Fig. 5E). Mutp53 levels also proportionally modulate the activated downstream EGFR signaling pathways PI3K and MAPK, which phosphorylate AKT and ERK, respectively (Fig. 5E). To show cooperative interaction between mutp53 and the EGFR pathway in mediating SC activities, we tested whether the modulation of mutp53 levels affects self-renewal ability of MDA231 cells upon...
EGFR inhibition. We found that EGFR inhibition by the dual EGFR/ErbB2 inhibitor lapatinib (40) at a low, cell viability preserving concentration (2 µmol/L; Fig. 5F) dramatically decreases mammosphere formation in MDA231 cells but not in MDA231 cells overexpressing R280K mutant p53 (left). Middle, representative images of low adherent culture (×10). G, inhibition of EGFR by high doses of lapatinib (10 µmol/L) has more profound effect on MDA231 cells while mutant p53 overexpression protects MDA231 cells from EGFR inhibition. CTB, cell viability assay. H, inhibition of EGFR signaling by lapatinib (24 hours with indicated concentrations) is partially rescued by mutp53 overexpression in MDA231R280K cells. Cells were serum starved overnight and then stimulated with EGF for 15 minutes before harvesting. GAPDH as a loading control.

**Figure 5.** (Continued.) F, overexpression of mutant p53 affects self-renewal ability of MDA231 cells upon EGFR inhibition. Lapatinib at low concentration (2 µmol/L), preserving cell viability (right), dramatically decreases mammosphere formation in MDA231 cells but not in MDA231 cells overexpressing R280K mutant p53 (left). Middle, representative images of low adherent culture (×10). G, inhibition of EGFR by high doses of lapatinib (10 µmol/L) has more profound effect on MDA231 cells while mutant p53 overexpression protects MDA231 cells from EGFR inhibition. CTB, cell viability assay. H, inhibition of EGFR signaling by lapatinib (24 hours with indicated concentrations) is partially rescued by mutp53 overexpression in MDA231R280K cells. Cells were serum starved overnight and then stimulated with EGF for 15 minutes before harvesting. GAPDH as a loading control.

EGFR inhibition. We found that EGFR inhibition by the dual EGFR/ErbB2 inhibitor lapatinib (40) at a low, cell viability preserving concentration (2 µmol/L; Fig. 5F) dramatically decreases mammosphere formation in MDA231 cells but not in cells overstuffed with R280K-mutant p53 (Fig. 5F). Also, inhibition of EGFR by high doses of lapatinib (10 µmol/L) has a more profound effect on MDA231 cells while mutp53 overexpression protects MDA231 cells from EGFR inhibition (Fig. 5G). These physiologic data correlate with higher EGFR level and signaling in mutp53 "overstuffed" MDA231R280K cells, which requires higher concentrations of lapatinib for EGFR inhibition, indicated by only partial inhibition of downstream of EGFR phosphorylated ERK at residue 202/204 in MDA231R280K cells (Fig. 5H).

In sum, our data imply that mutp53 expands the pool of mammary progenitors and breast cancer stem cells, which, at least in part, is mediated by EGFR family (EGFR and ErbB2) signaling.

**Discussion**

The p53 protein plays a key role in tumor suppression supported by the observation that p53 is mutated in over half of all human cancers of different origins. In contrast with other tumor suppressors, which are typically inactivated by gene silencing, deletion or nonsense mutations, more than 75% of all p53 mutations are missense mutations suggesting a selective advantage for retaining the mutant allele over simple loss of wt p53 functions. It is generally accepted that mutp53 may fuel carcinogenesis by exerting a dominant-negative effect over wt p53 protein, as well as by manifesting novel, oncogenic gain-of-function (GOF) activities (24). The best in vivo proof of mutp53 GOF is mutp53 knockin (KI) mouse models. All three originally reported mutp53 KI mice (R175H, R273H, and R248W) manifest GOF with a broader tumor spectrum, including adenocarcinomas, higher tumor bulk with increased grade and invasion, and novel metastatic ability compared with the p53-null allele (20, 22, 26, 41). A recent report on a new mutp53 R248Q KI mouse model showed the strongest GOF phenotype, reflected by earlier tumor onset and shorter survival compared to p53 null littermates, a phenotype not seen in the three previously reported KI models. Importantly, accelerated tumorigenesis in mutp53 R248Q mice was associated with expansion of normal hematopoietic and mesenchymal progenitors (10). Consistent with this data, a similar expansion of mouse mammary epithelial SCs in the Wnt-1 mammary mouse model was observed when a human mutp53 R175H allele was present compared with the p53 null allele (11). The notion that mutp53...
may have a positive role in regulating the mammary SC population is further supported by strong correlative clinical data. Compared with wtp53 breast cancer, the presence of mutp53 in primary human breast cancer tissues correlates with SC transcriptional signatures (42). Furthermore, poorly differentiated grade 3 breast cancers display the highest SC content and frequency of p53 mutations (43). In addition, statistical analysis of human wtp53 and mutp53 breast cancer tissues show a correlation between p53 mutations and concomitant acquisition of stem-like properties (43). Although these observations suggest a novel and intriguing oncogenic role of mutp53 in stem cell biology, the underlying mechanisms of this phenomenon remain to be defined.

To study the novel tumorigenic properties of mutp53 in breast cancer in vivo, we generated a mutp53 mouse model that harbors the MMTV-driven ErbB/Neu/Her2 transgene (1g), which drives mammary tumor formation. Strong clinical evidences of oncogenic cooperation between mutp53 and Her2 in human breast cancer determined our choice to use the MMTV-ErbB2/Neu mouse model. There is extremely high incidence (>70%) of Her2-positive breast cancer in LFS women compared with 25% of sporadic breast cancer (13), a high frequency of mutations in p53 specifically in Her2-positive breast cancer compared with other subtypes (72%; ref. 1), and mutations in the p53 gene indicate poor prognosis in Her2-positive breast cancer due to more aggressive disease (4, 5). A double transgenic mouse was made in a previous attempt to model mutp53; Her2-positive breast cancer by combining a Whey Acidic Protein (WAP)-promoter driven mutp53 Tg with the MMTV-ErbB2 Tg (44). However, this model had significant limitations, such as expression of mutp53 from an unknown genomic locus and expression restricted to when the WAP promoter is active, which is only in late pregnancy (44). Our improved mutp53 R172H;ErbB2 model has overcome these limitations. The mouse R172H p53 mutation corresponds to the R175H p53 mutation in humans, which frequently occurs in Her2-positive breast cancer (1) and therefore closely mimics the mutp53 subtype of human Her2-positive breast cancer and LFS.

Our newly generated p53 R172H;ErbB2 mouse model closely recapitulates the clinical data observed in HER2-positive breast cancer patients. Similar to humans, we found strong evidences for mutp53 and ErbB2 oncogenic cooperation. In contrast with "straight" p53 R172H KI mice (20, 22), p53H−/−;ErbB2 animals show accelerated mammary tumorogenesis as reflected by earlier tumor onset and shorter survival compared with p53−/−;ErbB2 counterparts (Fig. 1A). The formation of multiple, simultaneously initiated, multicentric tumors was significantly higher in the presence of the mutp53 allele, which most likely underscored this phenotype (Fig. 1B).

Because the presence of SCs can attribute to tumor initiation (7), we hypothesized that the observed phenotype is the physiologic consequence of an expansion of mammary SCs and/or mammary progenitors by the mutp53 allele. Functional analyses provided further support for this idea. First, mutp53 containing MECs displayed increased mammosphere formation ability (Fig. 2C) and higher expression of the SC marker ALDH1 (Fig. 2B). Second, pregnancy further increased mammary tumor multiplicity reaching the highest number in the presence of mutp53 (Fig. 2D), suggesting that mutp53 could be involved in expansion of a pregnancy responsive population of mammary progenitors (PI-MECs), which are thought to be the most important targets of ErbB2-induced mammary tumorogenesis (6, 32). Finally, mutp53 is highly stabilized in metastatic lesions of p53H−/−;ErbB2 mice in contrast with the heterogeneous staining found in primary mammary tumors (Fig. 2E). This suggests that tumor cells with the most stabilized mutp53 have a higher survival potential and/or higher invasive properties, possibly via induced EMT. EMT, a developmental program that confers mesenchymal cell traits on epithelial cells, has recently been linked to the mammary SC state in cancer, which leads to metastasis (34, 45). It was previously shown that depletion of mutp53 decreases invasion in vitro (18, 38, 39, 46) and metastases in mice (39, 46). Consistent with this idea, it was reported that the presence of mutp53 in the mammary tumors of bi-transgenic WAP−/−;WAP-mutp53 mice (but not in WAP−/− monotronagens, which express the SV40 T/αT antigen under the WAP-promoter) induces a specific oncogenic EMT gene signature (47). We observed a change of cell morphology from epithelial in p53−/−;ErbB2 MECs to mesenchymal in the presence of the mutp53 allele (Fig. 2F), indicating mutp53 MECs may be more invasive.

Recently, cancer SCs have been invoked as the seed for distant metastases, which are ultimately responsible for end-stage disease and death (7). Numerous reports suggest a functional link between EMT and cancer SCs (7, 34, 43, 45). The concept of metastases as ‘migrating cancer stem cells’ has been recently proposed (45). Selection of cells with highly stabilized mutp53 in metastatic lesions implies that high levels of mutp53 induce the acquisition of SC-like properties and promotes metastatic seeding to distant organs. In an attempt to identify the signaling pathways leading to expansion of TICs in the presence of mutp53, we found that mutp53 tumors express the highest levels of ErbB2 and exhibit increased ErbB2 signaling compared with p53 null counterparts (Fig. 4). According to recent reports, ErbB2 signaling drives carcinogenesis, at least in part, through regulation of the mammary stem/progenitor cell population. First, the TIC population of Her2-overexpressing breast cancer cell lines express the highest Her2 levels (15) and are directly responsive to Her2 inhibitors (14, 15). Lapatinib, a dual ErbB2/EGFR inhibitor, depletes the cancer SC pool from tumors of Her2-positive breast cancer patients (16). Second, expression of ErbB2 in murine MECs increases the cancer SC pool and induces mammary tumorigenesis, indicating a functional link between ErbB2-mediated expansion of cancer SCs and breast cancer development (14). Therefore, mutp53-mediated amplification of ErbB2 signaling may mechanistically underlie the expansion of mammary SCs that can lead to a more aggressive disease.

The question arises as to how mutp53 amplifies ErbB2 signaling mechanistically. To overcome proteotoxic stress inherent to malignant transformation, cancer cells induce a range of adaptive mechanisms, where the master transcription factor heat shock factor 1 (HSF1)-orchestrated heat shock response plays an essential role (48). Our previous in vitro studies identified a novel mutp53−/−HSF1 feed-forward loop, which provides cancer cells superior survival (37). We found that mutp53, via constitutive stimulation of EGFR and ErbB2 signaling, hyperactivates the MAPK and PI3K cascades to induce stabilization and transcriptional phosphoactivation of HSF1 on Ser326. Moreover, mutp53 protein has direct interaction with activated p-Ser326 HSF1 to facilitate HSF1 recruitment to its specific DNA binding elements and stimulate transcription of heat shock proteins, including Hsp90. In turn, induced Hsp90 stabilizes its oncogenic clients, including EGFR, ErbB2, and mutp53, thereby further reinforcing oncogenic signaling (37). Thus, mutp53 initiates a feed forward
loop leading to HSF1-mediated protein stabilization of mutp53 itself and EGFR family members, EGFR, and ErbB2. Importantly, here we confirm this axis in vivo and defined its physiologic consequences in a ErbB2-driven breast cancer mouse model. Importantly mutp53 alone can expand the luminal progenitor pool even in the absence of ErbB2 (Fig. 2A), similar to its role in hematopoietic and mesenchymal progenitors (10). Hence, mutp53 can exert its stem cell promoting activity by (i) amplification of ErbB2 signaling and/or (ii) via ErbB2-independent mechanisms, like amplification of EGFR and/or possibly other growth factor receptors signaling.

The other physiologic outcome of mutp53-mediated stimulation of ErbB2 signaling in vivo, which may not relate to SC biology, is enhanced proliferation of mammary cells. Breast cancer is thought to arise from MECs found in acinar structures, which are composed of terminal ductal lobular units that comprise a single layer of polarized luminal epithelial cells surrounding a hollow lumen (49). Activation of ErbB2 induces proliferation of acinar cells altering its epithelial architecture, which is typical for early stages of mammary tumorigenesis (35). Indeed, we found that mutp53 potentiates the proliferation of ErbB2-expressing cells in both murine mammary and human breast cancer (Fig. 3). Therefore, enhanced proliferation of luminal cells in the presence of mutp53 may fuel the initiation of premalignant lesions in the ErbB2 context, leading to increased tumor multiplicity observed in p53H+/−;ErbB2 mice. In support, mutp53 depletion from EGFR-expressing MDA468 human breast cancer cells was shown to induce reversion from highly disorganized to acinus-like structures with a hollow lumen in 3D culture (50).

In summary, our findings provide evidence that mutp53 cooperates with EGFR family EGFR and ErbB2 and amplifies their signaling, thereby inducing proliferation of mammary cells and expansion of mammary stem cells. Thus, our studies identify mutp53 as a key player and potential target in Her2 and EGFR-positive breast cancer.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

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**References**

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