Somatic Mutation of p53 Leads to Estrogen Receptor α-Positive and -Negative Mouse Mammary Tumors with High Frequency of Metastasis

Suh-Chin J. Lin,1,2 Kuo-Fen Lee,4 Alexander Yu. Nikitin,5 Susan G. Hilsenbeck,8 Robert D. Cardiff,7 Aihua Li,1,2 Keon-Wook Kang,1,3 Steven A. Frank,3 Wen-Hwa Lee,2 and Eva Y-H. P. Lee1,2

1Departments of Developmental and Cell Biology, 2Biological Chemistry, and 3Ecology and Evolutionary Biology, University of California, Irvine, California; 4The Salk Institute for Biological Studies, La Jolla, California; 5Department of Biomedical Sciences, Cornell University, Ithaca, New York; 6Department of Medicine and Department of Molecular and Cellular Biology, Breast Center, Baylor College of Medicine, Houston, Texas; and 7Center for Comparative Medicine, University of California, Davis, California

ABSTRACT

Approximately 70% of human breast cancers are estrogen receptor α (ERα)-positive, but the origins of ERα-positive and -negative tumors remain unclear. Hormonal regulation of mammary gland development in mice is similar to that in humans; however, most mouse models produce only ERα-negative tumors. In addition, these mouse tumors metastasize at a low rate relative to human breast tumors. We report here that somatic mutations of p53 in mouse mammary epithelial cells using the Cre/loxP system leads to ERα-positive and -negative tumors. p53 inactivation under a constitutive active WAPCrest (Clontech, Palo Alto, California) and subcloned 5

INTRODUCTION

Breast carcinogenesis requires multiple genetic changes including inactivation of tumor suppressor genes and activation of oncogenes (1). Mutations in p53 are observed in close to half of human cancers including breast carcinomas (2). p53 is also mutated in families with Li-Fraumeni syndrome in which early-onset female breast cancer is the most prevalent type of tumor (3). p53 is a transcription factor that regulates genes critical for cell cycle arrest and for apoptosis after genotoxic stress, thus preventing genome instability (4–6). In addition, amplification and/or overexpression of c-myc, Her2/Neu/erbB2, and cyclin D1 oncogenes are seen in a significant portion of breast cancers (2, 7).

p53 knockout mice are cancer prone and develop early-onset lymphoma and sarcoma (8, 9) but rarely mammary tumors (10) because of early mortality. To circumvent this problem, p53+/null mammary epithelium is transplanted into the fat pad of wild-type recipients and leads to the formation of breast tumors (11). Although this offers a potential model to study breast tumors, the influence of the transplantation process on carcinogenesis is not clear. Also, the transplanted cells are p53+/null, whereas in human tumors somatic mutations are acquired in a subset of cells during tumor progression. Previously, Jonkers et al. (12) reported that no mammary tumor formation was observed in p53 conditional-mutant mice carrying K14Cre transgene. Therefore, conditional inactiva-

tion of p53 in mouse mammary epithelial cells is necessary to generate a mouse model mimicking human carcinogenesis.

In addition to genetic mutations, steroid hormones play a critical role in breast carcinogenesis (13). About 70% of human breast cancers are estrogen receptor α (ERα)-positive and estrogen-dependent (14), and ERα and progesterone receptor (PR) expression is an important indicator of potential responses to hormonal therapy (15). However, the factors that control ERα expression in tumor cells are unknown. Thus far, most established mouse models seldom produce ERα-positive mammary tumors (16). In a C3(1)/SV40 T-antigen-transgenic model, ERα expression decreases during early mammary tumor progression (from low- to high-grade mammary intraepithelial neoplasia and becomes undetectable in invasive tumors (17). In Brca1- and Brca2-linked mammary tumors, the majority of tumors show no detectable ERα expression (18–20).

In humans, breast cancers frequently metastasize to other organs such as liver, lung, and specifically bone (21). Metastasis rather than primary tumors are responsible for most cancer mortality (21, 22). Less than 5% of patients with metastatic breast cancer have a long-term remission after treatment (22). In established mouse mammary tumor models, the tumor cells infrequently colonize other organs. Only 10% Brca1 tumors (18), 10% pten-/ tumors (23), and 0% Brca2 tumors (12, 20) metastasize. Many mouse mammary tumor viruses (MMTV/oncogene-bearing transgenic mice have a rare occurrence of metastasis (24), but metastatic tumors are observed in polyomavirus middle T antigen and neu protooncogene transgenic mice (25, 26).

We have generated a mouse breast tumor model by using Cre/loxP method to specifically inactivate p53 in mammary epithelial cells. This conditional inactivation of p53 leads to ERα-positive and -negative mammary tumors with a high rate of metastasis. We found that p53 inactivation during specific developmental stages critically determines ERα expression in mammary tumors. This breast tumor system provides a close model of the human disease and will be useful for both mechanistic and therapeutic studies of ERα-positive breast cancer.

MATERIALS AND METHODS

Targeting Vector Construction. A 10.75-kb clone covering exons 1–10 of p53 was isolated from a 129sv mouse genomic library. The 3.4-kb Xhol-HindIII fragment containing exons 2–9 was subcloned into pBR322. A replacement-type targeting vector was made by inserting the first loxP site into a BamHI site located in intron 6. A neo-cassette flanked by two loxP sites was inserted into the PvuII site in intron 4. The resulting construct was cleaved with Xhol and HindIII, blunt-ended and subcloned into p2TK (27). The finished targeting construct is designated p53neo/loxP2TK.

MMTVcre Transgene Construction. The backbone of the MMTVcre transgene is pBSpcKCR3 (28) containing part of the rabbit β-globin gene (the end of exon 2, intron 2, and exon 3), and the polyadenylation site. A 1.6-kb BgII-HindIII fragment composed of the Cre transgene with a nuclear localization signal was inserted within exon 3 of the globin gene of pBSpcKCR3. A 1.5-kb HindIII-Nhel fragment containing the MMTV-LTR was cut from pMAM (Clontech, Palo Alto, California) and subcloned 5’ to exon 2 of the globin gene. The finished transgene construct is designated pMMTVcre.

MMTVcre-Transgenic Mice Production. The 4.3-kb Xhol fragment containing the MMTVcre transgene was excised and purified. The transgenic

Received 11/10/03; revised 1/28/04; accepted 3/2/04.

Grant support: National Cancer Institute mouse consortium Grant CA04964, DOD BC013020, NIH CA4244, Breast Cancer Research Foundation to E. Lee and NC1P30 CA93373 to R. Cardiff.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Eva Y-H. P. Lee, University of California-Irvine, Sprague Hall, Room 140, 839 Health Science Court, Irvine, CA 92697-4037. Phone: (949) 824-9766; Fax: (949) 824-9767; E-mail: elee@uci.edu.
founders were generated by microinjecting the MMTVCre transgene fragment into the male pronucleus of fertilized eggs derived from CB6F1 × C57BL/6 intercrosses. Transgenic founders were identified by Southern analysis or PCR of tail DNA. The primers for the Cre transgene (363-bp amplified) were Cref (5'-GGTGCACATTATCGACGGTACA-3') and Cref (5'-CGGATCCGG- CGCATAACCGTG-3'). All mice were maintained in accordance with the guidelines of the Laboratory Animal Research of The University of Texas Health Science Center at San Antonio and Institutional Animal Care and Use Committee of University of California, Irvine.

**Generation of Conditional p53 Mutant Mice.** J1 embryonic stem cells were electroporated with Sall-linearized p53neoIox2TK and selected with G418 and 1-(2-deoxy-2-fluoro-D-β-arabinofuranosyl)-5-iodouracil. Embryonic stem cells harboring homologous recombination were identified by Southern blotting using a 3' probe external to the targeting region. The neo-cassette was removed from targeted embryonic stem cells by transient expression of p52Cre. Of 231 clones analyzed by Southern blotting, two contained a recombination that removed only neo. These two clones were expanded and injected into C57BL/6 blastocysts. Chimeric males were mated with C57BL/6 females, and germine transmission of the mutant allele was verified by Southern and PCR analyses. Subsequently, p53<sup>fp/fp</sup>/MMTVCre mice were generated by crossing p53<sup>fp/fp</sup> mice with MMTVCre mice. For PCR analysis, the following primers were used: primer x (5'-TGCGGA- CACGCAAAGCTGTAA-3'); y (5'-GCTCGAGGTCACAGCCTAG-3'); z (5'- CATGTCAGGACTCTAAC-3'); and p (5'-TACTCTCTCCCTCTTAATAAGCTAT-3'). Primers y and z flank the loxP site in intron 6 and amplify a 119-bp fragment from wild-type p53 and 188 bp from the FP allele. Primer pair x/z amplifies a 500-bp fragment from the deleted allele after Cre-mediated recombination. Primer pair p/q amplifies a 327-bp product in the wild-type allele as well as a 253-bp fragment for the pseudogene.

**Histology and Immunohistochemistry.** Tumor cells were collected in fixed 4% paraformaldehyde and processed through paraffin embedding following standard procedures. Sections were stained with H&E for histopathological evaluation. Immunostaining was performed following the protocol described in the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). For antigen retrieval, slides were heated for 20 min in 10 mM citrate buffer (pH 6.0) in a microwave oven. The antibodies used were CK8 and CK14 (1:2,000 and 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA), PR and Neu/erbB2 (500; Affinity Bioreagents, Golden, CO; MC-20; Santa Cruz Biotechnology, Santa Cruz, CA), PR and Neu/erbB2 (500; Affinity Bioreagents, Golden, CO; MC-20; Santa Cruz Biotechnology, Santa Cruz, CA), and p53 (1:2,000; CMS; Nova- castra Laboratories, Newcastle, United Kingdom).

**Western Analysis and Fluorescence Microscopy.** Tumor cells were grown in DMEM/F12 medium containing 15% fetal bovine serum, 10 mg/ml epidermal growth factor, and 1 μg/ml insulin. Cell lysates were prepared using EBC (50mM Tris-HCl, 120 mM NaCl, 1 mM EDTA, pH 8.0, 50 mM NaF, 0.5% NP-40) buffer, and lysates (50 μg) were separated by 10% gel electrophoresis and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with anti-ErbA (1:1,000, MC-20; Santa Cruz Biotechnology), anti-ErbB (1:1,000, PAI-31; Affinity Bioreagents, Golden, CO) or anti-actin (1:20,000; Sigma, St. Louis, MO) antibodies, followed by incubation with horseradish peroxidase or alkaline phosphatase/nitroblue tetrazolium secondary antibodies, and developed using an enhanced chemiluminescence (ECL) or 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution. Tumor cells were infected with Ad-25ERE-GFP adenoviruses at a multiplicity of infection of ~100. Cells were precultured in serum-free DMEM/F12 for 1 day and treated with 10 mM 17-β estradiol. Green fluorescent protein fluorescence was detected 24 h after 17-β estradiol treatment.

**RESULTS**

**Generation of the Floxed p53 Allele.** We engineered the floxed p53 allele (designated p53<sup>fp</sup>) by inserting the lox<sup>P</sup> sites into introns 4 and 6 of p53 through a two-step process (Fig. 1, A and B). The phosphoglycerate kinase neomycin resistance cassette was subsequently removed by transient expression of Cre recombine. Deletion of exons 5 and 6 led to an in-frame deletion of 99 codons (codons 123–221) that encode part of the DNA-binding domain. The presence of lox<sup>P</sup> sites in introns 4 and 6 did not interfere with the transcription of p53, and the full-length p53 protein was detected in p53<sup>fp/fp</sup> mouse embryonic fibroblasts in the absence of Cre recombine (Fig. 1C). A smaller protein product of expected mass of 39 kDa, designated p53<sup>fp/pfp</sup>, was detected in p53<sup>fp/fp</sup> mouse embryonic fibroblasts infected with Cre adenoviruses (Fig. 1C). IR-induced responses demonstrate that p53<sup>fp/pfp</sup> is transcriptionally inactive and fails to increase p21 target gene transcription. In contrast to wild-type p53, mutant p53<sup>fp/pfp</sup> protein was not stabilized after IR (Fig. 1D). No p53 protein was detected from mouse embryonic fibroblasts derived from p53<sup>fp/fp</sup> (p53<sup>−/−</sup>) mice (Fig. 1. C and D).

**Characterization of Cre-Transgenic Mice.** To introduce somatic p53 mutation in the mammary gland, different lines of mice expressing Cre under the control of MMTV-long terminal repeat, MMTVCre<sup>a</sup>, and MMTVCre<sup>b</sup> (collectively termed MMTVCre) were generated. In addition, to express Cre specifically in mammary luminal epithelial cells, WAPrtTACre-transgenic mice in which Cre is regulated by the whey acidic protein (WAP) promoter were also used (31). In the MMTVCre<sup>a</sup> line, Cre activity was restricted to the mammary gland, whereas in MMTVCre<sup>b</sup> and WAPrtTACre lines, Cre-mediated deletion was observed in many tissues (Fig. 2A). In contrast to the founder mice, when the WAPrtTACre transgene was bred to floxed p53 or reporter mice (see below), its activity became independent of doxycycline and pregnancy, likely attributable to modification of the transgene, and/or the unstable nature of the multicopy transgene. The altered transgene will be referred to as WAPCre<sup>a</sup> hereafter.

Because transgene activity varies depending on promoter and insertion sites, Cre-transgenic mice were crossed with R26R mice expressing LacZ after the removal of the floxed stop sequence by Cre (29) to identify the cell types targeted by Cre-mediated recombination in the mammary gland (Fig. 2B; Table 1; data not shown). In nulliparous MMTVCre<sup>a</sup> mice; R26R mice, LacZ was detected in ~0.7% cells of mammary gland. The expression remained low during 1st pregnancy and reached ~2.9% at 2nd pregnancy in both luminal epithelial and myoepithelial cells but not in stromal fibroblasts and adipocytes. In MMTVCre<sup>b</sup>; R26R mice, LacZ expression was also found in 5.6% of those cells in 2-week-old and ~20% in 7-week-old nulliparous mice. The expression increased to >60% in pregnant mice. The increased percentage of targeted cells in multiparous mice is expected, because MMTV promoter activities are up-regulated during pregnancy. In WAPCre<sup>a</sup>; R26R mice, LacZ activities were robust with 66% positive cells in 1-week-old and >90% in 17-day-old and 6-week-old nulliparous and pregnant mice. Thus, there are significantly higher numbers of targeted cells in WAPCre<sup>a</sup> than in MMTVCre mice during prepubertal/pubertal stages.

**Effects of p53 Inactivation on Spontaneous Mammary Carcinogenesis.** As expected, these mice develop tumors with different spectrums. The most common tumor type is mammary tumor, and the majority of mammary tumor-bearing mice (60–80%) had multiple primary mammary tumors (Fig. 3; Table 2). Mammary tumor latency varied depending partly on numbers of targeted cells and parity of mice. Nulliparous p53<sup>fp/fp</sup>/MMTVCre<sup>a</sup> females developed mammary tumors between 14 and 24 months of age with a median tumor latency (MTL) of 17.5 months with almost complete penetrance (23 of 24 mice), whereas all multiparous p53<sup>fp/pfp</sup>/MMTVCre<sup>b</sup> mice developed mammary tumors with a significantly shortened latency, between 11.5 and 24 months of age with a MTL of 15.5 months (P = 0.004, generalized Wilcoxon statistic). Both larger numbers of targeted cells as well as pregnancy-mediated cell proliferation could contribute to the shortened MTL. Nulliparous p53<sup>fp/fp</sup>/MMTVCre<sup>b</sup> females developed mammary tumors between 6 and 14 months of age with a MTL of 10.5 months in nearly 50% of mice (9 of 19 mice), whereas
multiparous p53<sup>fp/fp</sup>MMTVCre<sup>b</sup> mice developed mammary tumors between 7 and 12 months of age with a MTL of 11 months in 60% of mice (12 of 20 mice). The MTL of p53<sup>fp/fp</sup>MMTVCre<sup>a</sup> mice is significantly shorter than that of p53<sup>fp/fp</sup>MMTVCre<sup>b</sup> mice (P < 0.001), indicating distinct mammary carcinogenesis kinetics in mice with 1 or 3% versus 20% of p53-mutated cells. However, there is no statistical difference in the median MTL between 20% targeted cells in nulliparous and 60% in multiparous p53<sup>fp/fp</sup>MMTVCre<sup>b</sup> mice (P = 0.29). The lower penetration of mammary tumors in p53<sup>fp/fp</sup>MMTVCre<sup>b</sup> was attributable to the presence of other tumor types leading to early death. In p53<sup>fp/fp</sup>WAPCre<sup>c</sup> mice, mammary tumors developed between 8 and 12.5 months of age with a MTL of 9.5 months with high penetrance (13 of 14 mice). Consistent with the findings in p53<sup>fp/fp</sup>MMTVCre<sup>b</sup>, increasing targeted cells to >90% did not shorten the MTL further (P = 0.29). Of note, only one 23.5-month-old multiparous heterozygous p53<sup>/</sup><sup>/</sup>p53<sup>fp</sup>MMTVCre<sup>a</sup> mouse developed palpable mammary tumor in a cohort of mice between 20 and 26 months of age (n = 12; data not shown). The apparent long tumor
Fig. 2. Characterization of Cre activity. A, PCR analysis of Cre-mediated excision of floxed p53 alleles in p53\textsuperscript{fp/fp}; Cre mice. Primer pair x/z amplified a \~500-bp product in the deleted allele and a 377-bp fragment for the pseudogene, and primer pair p/q amplified a 327-bp product in the wild-type allele as well as a 253-bp fragment for the pseudogene. Tissue DNA samples were derived from p53\textsuperscript{fp/fp}; MMTV\textsuperscript{Cre}\textsuperscript{a} (a), p53\textsuperscript{fp/fp}; MMTV\textsuperscript{Cre}\textsuperscript{b} (b), and p53\textsuperscript{fp/fp}; WAP\textsuperscript{Cre}\textsuperscript{c} (c) mice. Nontransgenic, non-Cre-transgenic mammary gland; Mg (1st), (3rd), (4th), and (parous), mammary gland of 1st, 3rd, 4th pregnancy and of a parous female. B, \beta\textsuperscript{-}galactosidase staining of cells in the mammary gland of Cre; R26R mice. a-c, mammary gland from MMTV\textsuperscript{Cre}\textsuperscript{a}; R26R mice, nulliparous (a), 1st pregnancy (b), 2nd pregnancy (c). Arrow in c indicates \beta\textsuperscript{-}galactosidase detection in a myoepithelial cell; d-f, mammary gland from MMTV\textsuperscript{Cre}\textsuperscript{b}; R26R mice, 2-week-old (d), nulliparous (e), 1st pregnancy (f). g-i, mammary gland from WAP\textsuperscript{rtTACre}; R26R mice, 1-week-old (g), nulliparous (e), 1st pregnancy (f). Counterstained with nuclear fast red. Original magnifications, \times 200.
latency suggests that the internally truncated mutant p53 protein expressed under the endogenous promoter might not work in a dominant-negative manner.

Mammary tumors in all groups of mice are heterogeneous including adenocarcinoma, myoepithelial adenocarcinoma, adenosquamous carcinoma, and spindle cell tumor (Fig. 4, A-D). The majority of tumors were poorly differentiated invasive adenocarcinomas that share the most histopathological similarity with human tumors. In addition to mammary tumors, a few p53<sup>+/−</sup>MMTVCre<sup>fp/fp</sup> mice also developed lymphoma caused by a very low level of Cre activity in the lymphocytes or a low spontaneous incidence in aged C57BL/6 × 129sv mice. On the other hand, other tumor types were found in p53<sup>+/−</sup>, MMTVCre<sup>fp/fp</sup> and p53<sup>+/−</sup>WAPCre<sup>fp/fp</sup> mice caused by a broader Cre expression pattern (Table 2; Fig. 2A).

Importantly, up to half of p53<sup>+/−</sup>MMTVCre<sup>fp/fp</sup> mice had mammary tumor metastasis either in lung and/or liver after gross examination (Table 2; Fig. 4, E and F). Metastatic mammary tumor foci were also detected in p53<sup>+/−</sup>MMTVCre<sup>fp/fp</sup> and p53<sup>+/−</sup>WAPCre<sup>fp/fp</sup> mice (Table 2).

**Table 1. Cre-mediated recombination in Cre; R26R mice**

<table>
<thead>
<tr>
<th>Cre transgene</th>
<th>Nulliparous/ pregnant</th>
<th>Site</th>
<th>X-gal positive cells (%) (mean ± SE)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMTVCre&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Nulliparous (d42)</td>
<td>End bud/duct</td>
<td>0.73 ± 0.37 (n = 12)</td>
</tr>
<tr>
<td></td>
<td>1st pregnancy</td>
<td>Alveoli</td>
<td>0.22 ± 0.12 (n = 12)</td>
</tr>
<tr>
<td></td>
<td>2nd pregnancy</td>
<td>Alveoli</td>
<td>2.85 ± 0.44 (n = 12)</td>
</tr>
<tr>
<td>MMTVCre&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Nulliparous (d50)</td>
<td>End bud/duct</td>
<td>18.97 ± 3.19 (n = 12)</td>
</tr>
<tr>
<td></td>
<td>1st pregnancy</td>
<td>Alveoli</td>
<td>56.71 ± 0.06 (n = 12)</td>
</tr>
<tr>
<td>WAPCre&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Nulliparous (d44)</td>
<td>End bud/duct</td>
<td>90.73 ± 0.02 (n = 12)</td>
</tr>
<tr>
<td></td>
<td>1st pregnancy</td>
<td>Alveoli</td>
<td>96.80 ± 0.02 (n = 24)</td>
</tr>
</tbody>
</table>

<sup>a</sup> IC, incomplete.
<sup>b</sup> SE, standard error.

<sup>c</sup> The number of mammary tumor bearing-mice had metastasis of mammary tumor origin.

Significantly, 40% of the p53<sup>+/−</sup>WAPCre<sup>fp/fp</sup> mice (n = 15) had both ERα- and PR-positive tumors (Fig. 4H), compared with 42 tumors from p53<sup>+/−</sup>MMTVCre<sup>fp/fp</sup> mice that were all ERα- and PR-negative (Fig. 4G). The percentage of ERα-positive cells in ERα-positive tumors is over 90% whereas in ERα-negative tumors, no ERα-positive tumor cells were seen. The expression of ERα in tumors from p53<sup>+/−</sup>WAPCre<sup>fp/fp</sup> but not p53<sup>+/−</sup>MMTVCre<sup>fp/fp</sup> mice was further confirmed by Western blotting analysis (Fig. 4L). In contrast to ERα, ERβ was expressed in all tumors (Fig. 4L and data not shown). The closely correlated expression of ERα and PR, a downstream target of ER, suggests that ERα is functional in ERα-positive tumors. To further test this notion, adenosviruses carrying green fluorescent protein regulated by estrogen-response elements (EREs; 32) were used to infect tumor cells prepared from p53<sup>+/−</sup>, MMTVCre<sup>fp/fp</sup> and p53<sup>+/−</sup>WAPCre<sup>fp/fp</sup> mice. After treatment with estradiol, green fluorescent protein-positive cells were detected in tumor cells from p53<sup>+/−</sup>WAPCre<sup>fp/fp</sup> but not p53<sup>+/−</sup>MMTVCre<sup>fp/fp</sup> mice (Fig. 4M). Thus, ERα in the ERα-positive p53<sup>+/−</sup>WAPCre<sup>fp/fp</sup> tumors is transcriptionally active.

Deregulation of ERα expression during the premalignant stages of human breast carcinogenesis has been reported (33). Correspondingly, an increase of ERα-positive cells as well as clusters of ERα-positive cells (Fig. 4, J-K), in contrast to singularly distributed ERα-positive cells in the normal gland, was observed in mammary intraepithelial neoplasia (34) but not in hyperplasia without atypia (Fig. 4I). Thus, similar to human breast cancer, there are multistep histopathological changes and alterations in the ERα expression pattern during the progression of mammary carcinogenesis in these models.

Frequent genetic changes and prognostic markers of human breast cancer have been identified. To test whether this mouse model parallels human breast cancer in these alterations, selected genes were...
examined (Fig. 5). Amplification or overexpression of c-myc proto-oncogene is frequently observed in human breast cancer (35). In this model, c-myc amplification is found in 35% of mammary tumors from p53fp/fp MMTVCre mice (Fig. 5A). About two-thirds of tumors from p53fp/fp MMTVCre and p53fp/fp WAPCre mice showed Neu/erbB2 overexpression by immunostaining and Western blot analysis using anti-Neu/erbB2 and phosphotyrosine antibodies (Figs. 4H and 5B). Overexpressed Neu/erbB2 appears to be active because tyrosine residues of the receptors are phosphorylated (Fig. 5B). In addition, the activity of matrix metalloproteinase (MMP), matrix metalloproteinase...
9 but not matrix metalloproteinase 2, was increased dramatically (Fig. 5C). Several prognostic markers [e.g., AKY1 (STK15), CDC25B, cyclin E2, and MCM6] identified recently through gene expression profiling in human breast cancer (36, 37) also showed enhanced expression in these tumors (Fig. 5D).

**DISCUSSION**

Mice in a C57BL/6 × 129sv background are normally resistant to mammary tumor development (38, 39), but loss of p53 leads to a nearly complete incidence of mammary tumor with a high rate of metastasis to lung or liver. As the number of p53-inactivated cells increases, the MTL shortens. MTL reaches a plateau when \( \geq 20\% \) of the cells are targeted, indicating that there is a limit to the rate of breast carcinogenesis. Because ERα-positive mammary tumors develop in prepubertal/pubertal but not in adult mice, the timing of p53 inactivation might be critical for determining ERα expression. Genetic changes (e.g., in c-myc and Her2/Neu/herB2) and prognostic markers [e.g., AKY1 (STK15), CDC25B, cyclin E2, and MCM6] associated with human breast cancer are also seen in these mouse tumors.

Despite the use of different promoters with Cre, both luminal epithelial and myoepithelial cells were targeted by Cre recombinase, as indicated by the Rosa26 reporter strain (29). Heterogeneous tumor types (adenocarcinomas, myoepithelial adenocarcinomas, adenosquamous carcinomas, and spindle cell tumors) were seen in all mouse strains (Fig. 4). The majority of tumors were poorly differentiated invasive adenocarcinomas, which were also the most similar to human tumors histopathologically. Nonmammary tumors were also found, probably attributable to Cre expression in other tissues (Table 2; Fig. 2A). About 50% of mammary tumors metastasize to lung or liver (Table 2). Both histopathology and microarray analyses (data not shown) support that lung lesions are indeed tumor metastases. The metastasis frequency correlated with neither specific Cre-transgenic line nor tumor latency, consistent with previous observations (40). This tumor system recapitulates the high frequency of metastasis seen in advanced human breast cancer. By contrast, the experience of some investigators indicated that most mouse tumor models represent an early nonmetastatic stage of tumor development (41). Lung metastasis was also found in p53\(^{-/-}\)-BALB/c mammary gland transplant models (42); however, the precise metastasis rate is not clear.

These mouse mammary tumors exhibit a pattern of mutation and gene dysregulation similar to human breast cancer. Amplification or overexpression of the c-myc and erbB2 proto-oncogenes is frequently observed in human breast cancer (7, 35). About 35% of these mammary tumors were found to have c-myc amplification and two-thirds showed erbB2 overexpression. Matrix metalloproteinase 9 and cell cycle regulators such as AKY1 (STK15), CDC25B, cyclin E2, and MCM6 were up-regulated in these tumor cells. This is consistent with the recent results showing that overexpression of these genes in human breast cancer reflects poor prognosis (36, 37). The high frequencies of c-myc amplification and overexpression of erbB2 and cell cycle regulators in p53-mutated tumors suggest that these genetic alterations have pivotal roles during tumor progression.

An inverse relationship between the number of targeted cells and MTL was observed. It is reasonable to assume that larger numbers of targeted cells are more likely to acquire critical genetic changes leading to tumor development with shorter latency. Indeed, similar genetic (e.g., c-myc) and gene expression changes (e.g., Neu/erbB2, AKY1, CDC25B, cyclin E2, and MCM6) were seen at high frequencies in tumors of both short and long latency. Intriguingly, when the number of targeted cells exceeded 20%, MTL did not shorten further. To explain this phenomena, we assume that cancer initiation depends in part on the accumulation of a certain number of key mutations (43). If the key rate-limiting process was simply the accumulation of one additional mutation, then after the average cell has gone through \( T \) rounds of cell division, the probability of obtaining the key mutation is \( N \mu T \), where \( N \) is the number of targeted cells with the predisposing mutation and \( \mu \) is the mutation rate per cell division. If there are two rate-limiting mutational steps, then the probability of obtaining both mutations is, from the gamma distribution, approximately \( N \mu T^2 \). If we suppose \( \mu \) is approximately \( 10^{-6} \), and there are about \( T = 10 \) rounds of cell division, then saturation would happen when \( N \) is of the order 10\(^6\), which is probably much higher than the actual number of cells at saturation. Thus, to explain the saturation kinetics, there are two alternatives. First, the mutation rate per cell division may be higher than \( 10^{-6} \). This may occur because mutations of p53 increase the mutation rate per cell division. Second, some precancerosis clonal expansion may be occurring, which would increase the number of rounds of cell division and therefore decrease the number \( N \) of initial predisposing target cells needed to achieve saturation. On the basis of results of the MMTV\(^{Cre}\) line, it can be concluded that a saturation kinetic is reached by targeting \( \sim 20\% \) of mouse mammary epithelial cells. In the WAP\(^{Cre}\) line, it is likely that a different population of mammary epithelial cells is targeted; however, the tumor kinetics is similar to that of MMTV\(^{Cre}\). In addition to the number of targeted...
cells, it is plausible that the tumor latency might also be reflective of the type of cells targeted. Because multiple cell types are targeted in the models described here, the contribution of cell numbers and types of cells cannot be clearly differentiated.

On the basis of microarray profiling analysis, human breast cancers can be classified into five subtypes (44). It is not clear whether these heterogeneous types are originated from different cells of the mammary gland or specific cancer-initiating cells are targeted and heterogeneity develops subsequently during tumor progression. Recent studies have revealed that a population of breast cancer cells possess stem cell-like properties (45). However, it remains to be studied whether cancer-initiating cells of ERα-positive and -negative tumors are different. In WAPCre mice both ERα-positive and -negative mammary tumors were found, although mutations of p53 in MMTVCre mice resulted in only ERα-negative tumors. Parity may not be relevant because ERα-positive tumors were found in both nulliparous and parous p53+/−/−WAPCre mice. It is plausible that ERα-positive stem cells, in addition to ERα-negative stem cells, are targeted in WAPCre mice. In contrast, in MMTVCre mice, only ERα-negative stem cells are targeted. Because nearly 90% of cells are LacZ-positive during second pregnancy in MMTVCre mice (data not shown), this might suggest that only a small population of cells give rise to ERα-positive tumors in these adult parous mice. The Cre transgenes are active at different developmental stages in WAPCre and MMTVCre mice. Whether developmental stages affect the abundance of ERα-positive progenitor cells is not clear. Because it is feasible to isolate ERα-positive epithelial cells from normal mammary glands and tumors (32), molecular mechanisms underlying ERα-positive and -negative mammary carcinogenesis can be systematically addressed using this model.

ACKNOWLEDGMENTS

We thank Drs. Frank Graham and Lawrence Chan for Cre adenoviruses, Jolene Windle for the plasmid pBSpKCR3, Korinelia Polyak for Ad-25ERE-GFP viruses, Robert Reddick for histopathological comments, and Steve Lipkin and Paul Wakenight for critical review of the manuscript. We appreciate the contribution of Dr. Nanping Hu in the early phase of the project and of Kathryn Bushnell, Meihua Windle for the plasmid pBSpKCR3, Kornelia Polyak for Ad-25ERE-GFP viruses, Laci Bieche I, Lidereau R. Genetic alterations in breast cancer. Genes Chromosomes Cancer 1995;14:227–51.


