Hormone Dependence in Premalignant Mammary Progression

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ABSTRACT

Human breast cancers that are estrogen receptor (ER) negative convey a poor prognosis for patient survival. A mouse model that mimics essential biological and genetic attributes of a subset of human breast cancer is the BALB/c p53-null mammary epithelium, in which deletion of the tumor suppressor gene p53 results in enhanced tumorigenic risk. The experiments reported herein examine the hormone dependence of premalignant mammary progression in this model. The p53-null normal mammary epithelium exhibits the same dependence as p53 wild-type mammary epithelium on ovarian hormones for growth. However, in contrast to p53 wild-type epithelium, estrogen and progesterone, singly or in combination, strongly enhance tumorigenesis in p53-null mammary epithelium. The removal of progesterone signaling by deletion of the progesterone receptor eliminates progesterone enhancement of tumorigenesis. The immortalized premalignant outgrowth lines, termed PN, possess different tumorigenic capabilities, but the majority of these lines showed a strong dependence on ovarian hormones for growth and tumorigenesis. Although these lines are highly ER positive, a large number of tumors arising from these lines were ER negative and grew when implanted in ovariectomized mice. As was the case for p53-null normal mammary cells, hormonal stimulation was a strong promoter for tumorigenesis in the premalignant outgrowth lines and, surprisingly, was much stronger than the chemical carcinogen 7,12-dimethylbenzanthracene. In summary, these results demonstrate that p53-null mammary cells, which generate a significant percentage of ER-negative tumors, are highly responsive to the absence or presence of ovarian hormones during the normal and premalignant stages. This model would appear an excellent one to test the effects of chemopreventive agents on the development of both ER-negative and ER-positive mammary tumors.

INTRODUCTION

There are numerous models of mouse mammary tumorigenesis (1, 2). These include traditional models in which the oncogenic stimulus is either the MMTV, a chemical carcinogen such as DMBA, radiation, or reproductive hormones (2) or the numerous transgenic and gene deletion models developed in the past 15 years (1). Each model provides unique and specific information on genes and signal transduction pathways that can induce and/or strongly promote murine mammary tumorigenesis and theoretically provide information relevant to subsets of human breast cancer. The exact relevance to human breast cancer remains to be determined for the vast majority of these models. A model characterized recently is the BALB/c p53-null mammary epithelium, where deletion of the tumor suppressor gene p53 results in enhanced tumorigenic risk (3, 4). The p53-null mammary epithelium progresses through ductal hyperplasia and DCIS before becoming invasive breast cancer (5). The ductal hyperplasias are immortal, exhibit high telomerase activity, and are aneuploid and ER positive. These properties are different from the biological and cellular properties of the traditional alveolar hyperplasias found in the MMTV, chemical carcinogen, and spontaneous models of mammary tumorigenesis (6). Interestingly, the tumors arising from both types of hyperplasias are predominantly ER negative.

A similar result has been found in tumors arising in two other transgenic mouse models, the SV40 large T antigen and the polyoma middle T antigen. In the SV40 large T antigen mice, the ductal hyperplasias are ER positive and start losing ER during the DCIS stage (7). In polyoma middle T antigen mice, the early hyperplastic lesions show diminished ER and absence of PR (8). The ER phenotypic type of the ductal hyperplasias is of interest because ductal hyperplasias in the human breast are uniformly ER positive, and a subset of human DCIS and invasive breast cancer is ER negative (9, 10).

The origin of ER-negative human breast cancer is unknown. It is plausible that ER-negative breast cancers may arise from a mammary epithelial cell that was ER negative during its normal phenotype or from an ER-positive cell in which ER was transcriptionally down-regulated during premalignant progression (10, 11). Current information does not favor either of the major hypotheses for the origin of ER-negative human breast cancer. There is very little evidence of mutational inactivation of ER. However, recent studies have demonstrated that ER can be transcriptionally down-regulated in human breast cancer cell lines (10, 12).

Because the p53-null mammary epithelial cell possesses an ER pattern that mimics the expression patterns found in subsets of human ductal hyperplasia, DCIS, and invasive breast cancers, we investigated the hormone dependence of normal, hyperplastic; and cancer stages. The results show that p53-null mammary cells exhibit the same properties of hormone dependence as p53 wild-type cells yet are highly sensitive to the tumor-promoting effects of ovarian hormones. Furthermore, the growth and tumorigenesis of p53-null premalignant cells are dependent on ovarian hormones, and these cells are ER positive, yet they develop tumors that are frequently ER negative. The p53-null cells provide a good model to determine the origin of ER-negative tumor cells.

MATERIALS AND METHODS

Mice. All donor and recipient mice were bred and maintained at Baylor College of Medicine. The donor mice were BALB/c p53 homozygous null, and the recipient mice were p53 wild type (3). All mice were maintained in a conventional mouse facility with food and water provided ad libitum, and the room temperature was set at 70°F. The animal facility is American Association of Laboratory Animal Care accredited.

Transplantation. Samples of normal mammary duct were isolated from mice at 8–10 weeks of age and transplanted into both cleared inguinal mammary fat pads of 3-week-old mice (3). The transplanted duct samples grow and fill the mammary fat pads in 6–8 weeks. Samples of immortalized ductal outgrowth were serially transplanted (5). These ducts similarly fill the fat pad in 6–8 weeks. Each experiment was set up using different donor mice aged 8–10 weeks of age. Although the p53 deletion is the same in all donor mice, the array of secondary alterations important for neoplastic development include both common and unique events. The consequence is that the tumorigenic capabilities of mammary gland fragments vary over a small range between donor mice in the same host environment. Thus, the T50 of two untreated groups in two different experiments may be different (e.g., 60 weeks versus 50 weeks). Thus, each experiment always has an untreated control.
group to assess the effect of a particular treatment. For all experiments, one to two fat pads were processed as whole mounts at 8 weeks to examine the growth and morphology of the outgrowth. One to two fat pads were also processed for standard histological evaluation. The remaining outgrowths were followed for up to 14 months to assess for tumorigenic potential and/or used for other assays as described below.

**Experiments.** Experiments 1–3 examined the effects of hormone manipulation on growth and tumorigenesis in p53-null normal mammary gland. Experiments 4–6 examined the effects of hormone manipulation on growth and tumorigenesis in serially transplanted premalignant mammary outgrowth lines that were recently characterized and reported in Ref. 5. In experiment 1, the effect of bilateral ovariectomy was assessed on growth potential of p53 wild-type and p53-null mammary gland. In this experiment, samples of p53 wild-type duct were transplanted into the cleared mammary fat pads on one side, and p53-null duct was transplanted on the contralateral side. The mice were ovariectomized at 5 weeks of age, and the samples were processed as whole mounts 6 weeks thereafter (13, 14). There were 8 mammary fat pads/group. In experiment 2, the effect of chronic administration of estrogen (estradiol-17B) and progesterone, administered singly or in combination, on the tumorigenic potential of p53-null mammary epithelial cells was assessed over a 14-month period. Estrogen (50 μg) and progesterone (20 mg) were prepared in silastic tubing and implanted at 6-week intervals for the length of the experiment (15). There were five groups of 14 mice each. The groups were as follows: untreated; ovariectomized; treated with estrogen alone; treated with progesterone alone; and treated with estrogen plus progesterone. Ovariectomy was performed at 8 weeks after transplantation, a time period when the transplants had completely filled the mammary fat pad. Mice with p53 wild-type mammary epithelium were not evaluated because previous experiments demonstrated that chronic hormone stimulation did not convey a tumorigenic stimulus over these time periods (3).

In experiment 3, the dependence on progesterone-induced signaling for tumorigenesis in p53-null cells was directly tested by using PRKO mice (16). For these experiments, (BALB/c × FVB)F1 recipient mice were transplanted with duct from p53−/−, PR−/−, or p53−/−, PR+/+ donors. The recipient mice received a pituitary isograft at 5 weeks of age or were maintained as virgins. The four groups of mice were palpated weekly for tumors for up to 60 weeks. The different genotypes of the donor cells were generated by breeding (BALB/c × FVB)F1 mice that were heterozygous for both genes.

In experiment 4, the effect of ovariectomy was examined on the growth of immortalized p53-null duct outgrowth lines. The same protocol as described in experiment 1 above was used to follow growth and tumorigenesis.

In experiment 5, the hormone dependency of p53-null mammary tumors arising in intact mice was assessed by transplantation of tumor fragments into adult syngeneic p53 wild-type mice. Samples (1 mm3) were transplanted s.c. versus a tertiary isograft depending on the tumor growth rate.

In experiment 6, the effects of prolonged hormone stimulation via a pituitary isograft was measured by Vernier caliper weekly for a period of 4–10 weeks, depending on the tumor growth rate. In experiment 6, the effects of prolonged hormone stimulation via a pituitary isograft versus that of a chemical carcinogen, DMBA, were examined in four immortalized ductal outgrowth lines, P11B, P2, PNSA, and P110. There were 20 transplants/treatment group. Mice were either left untreated, received a pituitary isograft under the kidney capsule at 5 weeks of age (14), or received 1 mg of DMBA by oral gavage at 8 and 9 weeks of age (17). The mice were palpated for mammary tumors weekly for 1 year.

**Immunohistochemistry.** Samples of the outgrowth lines and the transplanted tumors were evaluated for hormone receptors (estrogen and progesteron) by standard methods as described in Ref. 16. The samples were fixed in cold 4% paraformaldehyde for 1 h before being processed for paraffin-embedded sections. The antibody to ER was SC-20, and the antibody to PR was SC-19 (Santa Cruz Biotechnology, Santa Cruz, CA). The negative control for ER was mammary preneoplastic outgrowth line TM2, which is ovarian hormone independent for growth and does not contain ER, and the negative control for PR was mammary gland from the PR-null mice. Some data on ER positivity in the immortalized ductal outgrowth lines were reported recently in Ref. 5. In the tumors, three separate samples per treatment group were examined for each tumor transplant, and 500 cells were counted per sample.

The scoring of ER- and PR-positive cells followed the semiquantitative system developed by Harvey et al. (18). The system scores both the frequency and intensity of ER (or PR) staining on a scale of 0–5 and 0–3, respectively, with the highest numbers representing the most positive ER values. In this scoring system, 0 represents a total absence of ER, 1 represents up to 1% of cells positive for ER, 2 represents between 1% and 10% of cells positive for ER, 3 represents between 11% and 33% of cells positive for ER, 4 represents between 34% and 65% of cells positive for ER, and 5 represents >66% of cells positive for ER. For intensity, 0 = negative staining for ER, 1 = weak staining for ER, 2 = intermediate staining for ER, and 3 = strong staining for ER.

**Statistical Analysis.** The results on percentage fat pad filled were evaluated statistically by two-sided Student’s t test. Results were considered significantly different at P < 0.05.

**RESULTS**

**Normal Mammary Gland.** Previous results demonstrated that the p53-null mammary epithelium exhibits normal growth and morphogenesis (3, 19). In experiment 1, the results indicate the p53-null mammary epithelium at 8–12 weeks posttransplantation exhibits a normal distribution and cellular localization of ER and PR (Fig. 1A and B). At 8 weeks posttransplantation, the ER score for wild-type and p53-null epithelium was 5–6 on a scale of 8, using the system described in Ref. 18. Additionally, the growth of the p53-null mammary epithelium was absolutely dependent on ovarian hormones because the cells did not exhibit expansive growth in ovariectomized mice (Fig. 2). The growth of p53-null mammary epithelium in ovariectomized mice was inhibited to the same extent as the growth of p53 wild-type epithelium (84% versus 77%, respectively; P > 0.05). Thus, in these assays as well as those reported previously, the p53-null normal mammary epithelium behaved similarly to the p53 wild-type normal mammary epithelium.

The growth of the mammary epithelium in ovariectomized mice produced one very interesting and unexpected result. The expression of ER was up-regulated in both wild-type and null mammary epithelium with >90% of the luminal epithelial cells exhibiting a very strong ER signal. The intensity of the signal was greater than that detected in mammary epithelium in intact mice (Fig. 1C). PR was present, but the levels were down-regulated (Fig. 1D) compared with that detected in p53-null mammary epithelium in intact mice.

**Hormone-induced Tumorigenesis.** Previous results demonstrated that hormones provided by a pituitary isograft (progesterone and prolactin) induce normal morphogenesis in p53-null mammary epithelium (3, 20). However, these hormones also enhanced the frequency of aneuploidy as well as mammary tumorigenesis (20). The results in Table 1 show that chronic levels of estrogen and progesterone, administered singly or in combination, markedly enhanced tumorigenesis compared with the untreated group. There were no significant differences in tumor incidences or tumor latencies among the three hormone treatment groups (P > 0.05). In contrast, ovariectomy at 5 weeks of age almost totally blocked tumorigenesis (Table 1) and resulted in a tumor incidence less than that in untreated mice (P < 0.05).

The marked dependence on progesterone-mediated signaling for tumorigenesis was tested directly by cross-breeding the p53-null mice with FVB PRKO mice to generate p53−/−, PR−/− mammary ducts. The results in Table 2 show that mammary tumorigenesis in hormone-stimulated p53-null epithelial cells was markedly reduced in the absence of progesterone signaling (i.e., when the PR was deleted) from 84% to 32%. In these mice, progesterone levels are high due to the prolactin secreted by the pituitary isograft. In virgin mice that have a low level of circulating progesterone, the absence of PR reduced mammary tumorigenesis in p53-null cells from 52% (13 of 25) with a TE50 of 52 weeks to 36% (9 of 25) with a TE50 of >52 weeks in the p53−/−, PR−/− group. In the absence of PR, there was no statistical difference in tumor incidence between the pituitary (8 of 25) and virgin (9 of 25) groups.
Premalignant Outgrowth Lines. We have recently described the biological and tumorigenic characteristics of a series of serially transplantable outgrowth lines derived from p53-null mammary epithelium (5). The majority of these lines, but not all of them, maintain expression of ER and PR. Table 3 shows the growth and tumorigenicity of some of these outgrowth lines in ovariectomized mice. Of the eight outgrowth lines examined, the growth of six cell lines (PN1A, PN1B, PN2, PN6, and PN10) was significantly decreased by the absence of ovarian hormones. These six cell lines all contained nuclear-localized ER and PR (5). Of the two hormone-independent lines, one line (PH1) did not contain ER, and the other line (PN7) contained normal levels of ER (5). The extent of branching was also markedly decreased in the mammary transplants growing in the ovariectomized mice.

Table 1  
Hormone-induced tumorigenesis in p53-null mammary epithelium

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of tumors/no. of transplants (%)</th>
<th>TE&lt;sub&gt;50&lt;/sub&gt; (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>11/26 (42)</td>
<td>&gt;62</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>2/26 (7.7)</td>
<td>&gt;62</td>
</tr>
<tr>
<td>Estrogen</td>
<td>17/26 (65.4)</td>
<td>38</td>
</tr>
<tr>
<td>Progesterone</td>
<td>24/28 (85.7)</td>
<td>37</td>
</tr>
<tr>
<td>Estrogen/progesterone</td>
<td>21/28 (75)</td>
<td>39</td>
</tr>
</tbody>
</table>

Table 2  
Tumorigenesis in p53-null and PR-deficient mammary epithelium

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of tumors/no. of transplants (%)</th>
<th>TE&lt;sub&gt;50&lt;/sub&gt; (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53&lt;sup&gt;−/−&lt;/sup&gt;, PR&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>13/25 (52)</td>
<td>52</td>
</tr>
<tr>
<td>p53&lt;sup&gt;−/−&lt;/sup&gt;, PR&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>9/25 (36)</td>
<td>&gt;52</td>
</tr>
<tr>
<td>p53&lt;sup&gt;−/−&lt;/sup&gt;, PR&lt;sup&gt;+/+&lt;/sup&gt;, pit.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21/25 (84)</td>
<td>41</td>
</tr>
<tr>
<td>p53&lt;sup&gt;−/−&lt;/sup&gt;, PR&lt;sup&gt;−/−&lt;/sup&gt;, pit.&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8/25 (32)</td>
<td>&gt;52</td>
</tr>
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<sup>a</sup> pit., pituitary isograft.
Although ovarian hormone responsive for growth, the preneoplastic ductal outgrowth lines grew better in ovariectomized mice than primary transplants of p53-null normal mammary epithelium. The mean growth of the six ovarian hormone-dependent lines was 58.6%, which was significantly greater than the 16% growth attained by the primary transplants (P < 0.05).

The absence of ovarian hormones also inhibited mammary tumorigenesis, regardless of the tumorigenic potential of the ductal outgrowth lines (Table 3). The highly tumorigenic line PN1A had a decreased tumor incidence (87% versus 50%) and median tumor latency (20 versus 24 weeks) in ovariectomized mice. The weakly tumorigenic lines PN1B and PN5A had decreased tumor incidences after 1 year of transplantation (25% versus 0% and 27% versus 0%, respectively). In this experiment, the weakly tumorigenic lines PN2 and PN10 did not produce any tumors in either control or ovariectomized mice. Line PN6, a moderately tumorigenic line, showed a marked increase in tumor latency as well as a decrease in tumor incidence. The absence of significant growth even after 12 months in ovariectomized mice was evident by inspection of whole mounts of the transplants from PN2. At the termination of the experiment, the transplants in the intact controls exhibited 100% fat pad filled (FPF) at 8 weeks posttransplant, whereas the transplants in the ovariectomized mice (n=8) attained an extent of growth (65% fat pad filled) similar to that seen in the 8 week transplants. The absence of ovarian hormones did not affect the tumorigenic potential of the hormone-independent outgrowth lines PH1 and PN7.

**Hormone Dependency of Tumors.** Ovarian hormone-dependent tumors are infrequent in the majority of mouse models of mammary cancer. Because both the normal p53-null mammary epithelium and the majority of the preneoplastic outgrowth lines are ovarian hormone responsive for growth, the hormone-dependent growth of tumors arising from the preneoplastic ductal outgrowth lines is ovarian hormone dependent. Of 27 tumors, only 3 (11%) were responsive to ovarian hormones for sustained growth. These three tumors grew 40%, 50%, and 58% of the mean size in ovariectomized mice compared with the growth in intact mice. All three tumors were positive for ER (30–50% positive). There were 23 tumors (85%) that grew equally well in ovariectomized mice and in intact mice. These 23 tumors were from lines PN1A, PN1B, PN5A, PN7, and PN9. Fifteen tumors of this tumor subset were examined for ER, and 14 were negative, whereas one showed 5% of cells positive for ER. This subset of tumors was not examined for PR. One of the 27 tumors (PN7) grew significantly better (5.8×) in ovariectomized mice than in control mice. This tumor was ER negative.

Two tumors arising from primary transplants of p53-null mammary epithelium in untreated mice were examined for hormone dependency, and both tumors grew as hormone-independent cell populations. Seven tumors arising from primary transplants of p53-null mammary epithelium in untreated mice were examined for the presence of ER, and all seven were negative for ER. Interestingly, in two of the seven tumors, areas of ductal hyperplasia and DCIS distinct from the invasive cancer were positive for ER and PR. Thus, the vast majority of the mammary tumors arising in p53-null mammary epithelium are negative for ER and grow as ovarian hormone-independent cell populations.

**Prolonged hormone stimulation significantly enhances tumorigenesis in primary transplants of p53-null normal mammary epithelium.**

Hormone stimulation resulted in 100% tumor incidence with a TE50 of 37 weeks compared with 62% tumor incidence with a TE50 of 50 weeks (3). We examined the relative tumorigenic potential of prolonged hormone stimulation versus a known chemical carcinogen, DMBA, on four of the immortal premalignant ductal outgrowth lines (Fig. 3). Interestingly, in three poorly tumorigenic lines (PN2, PN5A, and PN10), prolonged hormone stimulation elicited by a pituitary isograft was significantly more tumorigenic than DMBA. Whereas prolonged hormone stimulation induced mammary tumors in 44 of 60 transplants (73%), DMBA only induced 8 tumors in 60 transplants (13%) in these three outgrowth lines. In contrast, the tumorigenic potential of the modestly tumorigenic line PN1B was only marginally increased by either hormone stimulation or DMBA. Tumors arising in outgrowth lines PN2 and PN10 were highly positive for ER and PR. At this time, we have not assessed hormone dependency by the functional assay of growth in ovariectomized mice.

**DISCUSSION**

The p53-null mammary epithelial model differs from the traditional mouse models of mammary tumorigenesis in several fundamental biological properties (5, 6). The traditional mouse models of mammary tumorigenesis, which are induced by mouse mammary tumor virus standard, arise from alveolar hyperplasias. These alveolar hyperplasias are negative for ER at the onset and generate ER-negative, hormone-independent tumors. In chemical carcinogen-treated mice, tumors arise from both alveolar hyperplasias and ductal hyperplasias. However, tumors are primarily hormone independent. Because the p53-null normal mammary epithelium and the serially transplanted, immortalized ductal outgrowths were ER positive, it was of interest to determine the hormonal dependency of preneoplastic progression in these cells.
The hormonal dependency of the p53-null normal mammary epithelium is indistinguishable from that of the p53 wild-type epithelium. Previous results demonstrated that growth and morphogenesis in hormone-stimulated mice are the same for the two p53 genotypes (19, 20). The results reported herein show that the same is true for growth in the absence of ovarian hormones. The effect of hormones on functional differentiation of the p53-null mammary epithelium has recently been examined by serial analysis of gene expression, and the results demonstrated normal levels of RNA expression of the major milk protein genes in the p53-null mammary epithelial cell (21). Thus, extensive examination of the p53-null normal mammary epithelial cell indicates that it possesses a normal pattern of hormone responsiveness and dependency.

In contrast to wild-type BALB/c normal mammary epithelium, p53-null mammary epithelium is highly susceptible to tumor induction by estrogen and progesterone, given singly or in combination. These same studies demonstrated that hormone stimulation provided by a pituitary isograft did not enhance mammary tumorigenesis in the p53 wild-type mammary epithelium. The importance of normal signaling of one of the ovarian hormones, progesterone, was demonstrated by removing PR function in the p53-null epithelial cell. Under the strong tumorigenic stimulus of a pituitary isograft in which progesterone levels are increased chronically 7-fold (22), the absence of PR reduced tumor development to levels below that observed in hormone-stimulated p53-null, PR wild-type cells. In the PR wild-type mice, circulating levels of progesterone are low but detectable (22), thus the results support the idea that blocking signaling mediated by low levels of just one ovarian hormone could decrease tumor risk. Blocking circulating levels of both ovarian hormones by ovariectomy (hence blocking signaling mediated by both estrogen and progesterone) was most effective and reduced tumorigenesis to almost zero. However, a few tumors eventually developed under these stringent hormone depletion conditions, indicating that tumorigenesis can occur in the presence of extremely low hormone levels.

The hormonal regulation of tumorigenesis in p53-null mammary cells is of interest for several reasons. First, the results indicate that ER-negative tumors possess a development stage that is highly hormone dependent. This result is in accord with results from human studies where the vast majority of mammary epithelial cells in ductal hyperplasias are ER positive, yet ER-negative cancers comprise about 30% of all breast cancers (9, 10). Similarly, the immortalized p53-null ductal outgrowth lines are frequently ER positive and frequently give rise to ER-negative tumors. Second, this model differs from two other transgenic models that generate ER-negative tumors. In the C3-SV40 large T model, estrogen supplementation promotes tumorigenesis; however, ovariectomy at 8 weeks of age had no significant effect on survival rate or tumorigenesis (7). This is in sharp contrast to the dramatic effect of ovariectomy on tumorigenesis in p53-null mammary cells. In MMTV-neu transgenic mice, hormone stimulation by parity promoted tumorigenesis, but ovariectomy at 16–20 weeks of age had no significant effect on tumorigenesis (median time of onset was 52 weeks of age; Ref. 23). Additionally, hormone stimulation by progesterone alone in ovariectomized neu mice also promoted tumorigenesis to a greater degree than that observed in intact neu mice (23).

Finally, tamoxifen treatment started in 24-week-old mice had no inhibitory effect on MMTV-neu transgenic mice and had a 50% inhibitory effect when started in 12-week-old MMTV-neu transgenic mice (24). A comparison of the three models suggests that p53-null mammary cells retain ovarian hormone sensitivity for a greater duration of their premalignant progression. Whether this is attributable to intrinsic differences in the molecular programming induced by different oncogenic agents in the three systems or to a difference in specific subsets of mammary cells affected by promoter-regulated expression of oncogenes compared with deletion of p53 in all mammary cells or is simply a reflection of the very rapid premalignant progression in the MMTV-neu and C3-SV40 large T mammary cells remains to be tested.

The results on the immortalized, serially transplanted ductal outgrowth lines complement and extend the results on the p53-null normal mammary epithelium. Recent results indicate that these lines have varying tumorigenic capability; some are highly tumorigenic, and some are weakly tumorigenic (5). The results reported herein indicate that the outgrowth lines show a degree of hormone responsiveness for both growth and tumorigenocity. Six of the eight out-
growth lines examined were ovarian hormone responsive for growth. Second, it is important to note that the dependence on hormones for growth was relatively less than that for p53-null normal mammary cell. Thus, the immortalized ductal outgrowth possessed a relaxed requirement for steroid hormones; however, this degree of dependence was sufficient to impact tumorigenic development, regardless of whether the line had high tumorigenic potential (i.e., PN1A) or low tumorigenic potential (i.e., PN1B, PN5). The molecular basis for the decreased dependence on steroid hormones for growth has not been examined. At this point, preliminary results show that the levels of the cell cycle-regulatory protein, cyclin D1, are increased in the PN immobilized outgrowth lines compared with the p53-null normal mammary cells.6 Future experiments examining cell cycle-regulatory proteins in p53-null cells grown in ovariectomized mice remain to be done.

As with p53-null normal mammary cells (25), the tumorigenic potential of these immortalized premalignant lines was enhanced to a much greater extent by hormone stimulation than by DMBA, a well-established mammary carcinogen. The mechanisms underlying the effects of steroid hormones on the p53-null normal mammary epithelium are not known, but current studies suggest that such mechanisms do not involve DNA damage directly, as measured by assays for oxidative damage, sister chromatid exchange, or cytogenetic changes.5 One explanation for the low level of susceptibility to DMBA might be attributed to the specific mammary cell type that comprises the p53-null ductal outgrowths. Previous studies have shown that immortalized mammary ductal outgrowths of BALB/c genotype and p53 wild type were refractory to DMBA-induced tumorigenesis (26, 27). However, the morphological progression from a ductal outgrowth to an alveolar outgrowth was correlated with a marked increase in susceptibility to DMBA (27). In the present experiment, a positive control was the alveolar hyperplastic outgrowth line TM10. This line produced 94% tumors (15 of 16) within 26 weeks of transplant, whereas the untreated control did not produce any tumors.

These results again raise the issue of the origin of ER-negative tumor cells. In this model, there were both ER-negative and ER-positive tumors. Thus far, the frequency of PR-positive cells is concordant with ER, although we have performed dual immunofluorescence studies to ascertain whether the same epithelial cell is expressing both ER and PR. The majority of the tumors measured for ER were derived from the immortalized transplantable premalignant outgrowth lines that were highly ER positive (e.g., PN1A, PN1B, and PN5A). These data do not provide a definitive answer to the issue of the origin of the ER-negative tumor cells. On one hand, the high percentage of ER-negative tumors arising from a predominantly ER-positive cell population argues for the idea that the ER-negative tumor cell may arise from an ER-positive hyperplastic cell. The ability to induce ER by epigenetic manipulation in ER-negative tumors further supports this idea (12). However, at this time, one cannot conclusively eliminate the possibility that ER-negative tumors arise from the ER-negative normal cell (10, 11).

In summary, the p53-null mammary epithelial cell model mimics the properties of a subpopulation of human breast cells that generate ER-negative cancers and provides a model to examine and test chemopreventive and chemotherapy modalities targeted against the ER pathway.

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