p16\textsuperscript{INK4a} and p53 Deficiency Cooperate in Tumorigenesis\textsuperscript{1}

Norman E. Sharpless, Scott Alson, Suzanne Chan, Daniel P. Silver, Diego H. Castrillon, and Ronald A. DePinho\textsuperscript{2}

Departments of Adult Oncology, Medicine and Genetics, Dana-Farber Cancer Institute and Harvard Medical School [N. E. S., S. A., S. C., D. P. S., D. H. C., R. A. D.], and Department of Pathology, Brigham and Women's Hospital, Harvard Medical School [D. H. C.], Boston, Massachusetts 02115

Abstract

The combined impact of mutations in p16\textsuperscript{INK4a} and p53 was examined in cellular growth, transformation, and tumor formation. In cultured cells, p16\textsuperscript{INK4a} loss enhanced growth at high density and conferred susceptibility to oncogene-induced transformation. In vivo, mice doubly deficient for p16\textsuperscript{INK4a} and p53 showed an increased rate of tumor formation with particular susceptibility to aggressive angiosarcomas. Furthermore, p16\textsuperscript{INK4a} silencing by promoter methylation was detected in tumors derived from p16\textsuperscript{INK4a}\textsuperscript{−/−} and +/+ mice, independent of p53 status. These data suggest at least one general feature of malignancy, resistance to density-mediated growth arrest depends on p16\textsuperscript{INK4a} rather than p53. This cooperation between p16\textsuperscript{INK4a} and p53 loss in tumorigenesis is consistent with the view that these genes function in distinct anticancer pathways.

Introduction

Loss of Rb and p53 pathway function occurs in most, if not all, human cancers. The Rb pathway can be perturbed in several ways, including D-type cyclin overexpression, Rb deletion, CDK4 point mutation or amplification, and p16\textsuperscript{INK4a} deletion, point mutation, or promoter silencing (1, 2). In the p53 pathway, loss of function is typically attributable to p53 point mutations or deletion, MDM2 amplification, or p14\textsuperscript{ARF} (p19\textsuperscript{ARF} in mice) deletion (1). Various combinations of these lesions occur in human cancers, but the combination of p53 and p16\textsuperscript{INK4a} loss appears most common, particularly in adult carcinomas (2, 3). A large body of genetic, epidemiological, and biochemical evidence has established these proteins as components of distinct barriers to human cancer. Loss of p53 has been suggested to fuel genomic instability, provide resistance to chemo-radiotherapy, and attenuate growth arrest in response to telomeric shortening, hypoxia, and nutrient deficiency (reviewed in Ref. 4). The tumor-specific stimuli that induce p16\textsuperscript{INK4a} expression, however, are less clear but may relate to the need to bypass the replicative senescence checkpoint (5), as well as the pressure to deactivated G1 phase control in the setting of suboptimal growth conditions (6, 7) or oncogene activation (8, 9). Early passage MEFs\textsuperscript{3} from the p16\textsuperscript{INK4a} -specific knockout mouse (p16\textsuperscript{INK4a}−/−) have been shown to possess similar growth kinetics compared with littermate wild-type control cultures when passaged at nonsaturating densities (10, 11). However, the major distinction of p16\textsuperscript{INK4a}−/− cultures was a greater ease of immortalization when passaged serially on a 3T9 protocol (11). This impact of p16\textsuperscript{INK4a} mutation on MEF biology was subdued, and we surmised that the impact of p16\textsuperscript{INK4a} loss would be more evident in the setting of combined tumor-relevant mutations. Toward that end, we sought to determine whether p16\textsuperscript{INK4a} loss conferred additional tumor-relevant capabilities in the setting of p53 deficiency.

Materials and Methods

Mouse Colony and Histopathology. Animals were generated as described previously (11). p16\textsuperscript{INK4a}−/− males (~75% FVB/n) were mated with p53\textsuperscript{−/−} females (n > 10 FVB/n; Ref. 12). Nonlittermate p16\textsuperscript{INK4a}−/− p53\textsuperscript{−/−} females were then mated with p16\textsuperscript{INK4a}−/− p53\textsuperscript{−/+} or p16\textsuperscript{INK4a}−/+ p53\textsuperscript{−/−} males to generate the experimental colonies (~87.5% FVB/n). Littermate controls were analyzed in all instances for tumor-free survival. Animals were genotyped by PCR and monitored as described previously (11, 12). Histological characterization was done as described previously (11). We did not perform immunohistochemical analysis on the majority of tumors in this work, and therefore, many tumors were classified as malignant spindle cell neoplasms. These tumors generally do not express markers of specific mesenchymal differentiation (Myf, S100, desmin, and CD31) yet most likely represent poorly differentiated sarcomas and are termed “fibrosarcoma” or “malignant fibrous histiocytoma” by others. It is possible that a minority of these tumors represent poorly differentiated squamous cell carcinomas, amelanotic melanomas (particularly given that this analysis was performed on albino mice), or other poorly differentiated malignancies.

Cellular Analysis. MEFs were generated from day 13.5 embryos and grown in DMEM with 10% FCS (HyClone), 50 μM 2-mercaptoethanol, and Pen/Strep. Growth curves were performed as described previously (11) using a VSV-G pseudotype retrovirus concentrated by centrifugation and encoding a dominant-negative mutant of human p53 (V143A). For modified 3T9 analysis, 9 × 10\textsuperscript{4} cells were passaged into either 6-, 10-, and 15-cm dishes every 3 days. Four independent lines were assayed per density. UV treatment and MSP of p16\textsuperscript{INK4a} were performed as described previously (11). For hypoxia experiments, 25,000 cells/well in 12-well plates were grown at 1% O\textsubscript{2}, and cell survival was measured at times indicated. For the high-density experiments, six replicate plates of 1 × 10\textsuperscript{6} cells (p16\textsuperscript{INK4a}−/− or +/−, with and without DNP53) were grown in 10-cm dishes; two lines per genotype were assayed. Cells were refed every other day, and two plates per genotype were harvested on the indicated days. Cells were counted using trypan blue, and cell cycle profile was determined by Propidium Iodide staining. Transformation assays were performed as described previously (11). In brief, early passage (passages four to six) littermate cultures (eight lines per genotype in five independent experiments) were transfected with H-Ras(G12V), and either p53-DD (M. Oren) or SV40 Large T-Ag (J. DeCaprio), and then refed but not passaged for 10 days.

Tumor Analysis. Primary tumors from 18 p53\textsuperscript{−/−} and 17 p53\textsuperscript{−/+} were analyzed; the distribution of p16\textsuperscript{INK4a} genotypes is shown in Table 2a. Western blotting for p53, Rb, p16\textsuperscript{INK4a}, and p19\textsuperscript{ARF} was performed as described on primary tumors (11). In brief, tumors were lysed in EBC (1:200 2A10, gift from A. Levine) or SV40 Large T-Ag (J. DeCaprio), and then refed but not passaged for 10 days.

1 To whom requests for reprints should be addressed, at Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street (M413), Boston, MA 02115. Phone: (617) 632-6085; Fax: (617) 632-6069; E-mail: ron_depinho@dfci.harvard.edu.

2 The abbreviations used are: MEF, murine embryo fibroblast; LOH, loss of heterozygosity; MSP, methylation-specific PCR; Rb, retinoblastoma; T-Ag, T antigen.
Results

p16<sup>INK4a</sup> Loss Enhances Growth at High Density, Regardless of p53 Status. To examine the effect of p16<sup>INK4a</sup> status on the growth of cells lacking functional p53, early passage MEF cultures were infected with a high-titer retrovirus encoding a dominant-negative mutant of human p53 (V143A). Comparable expression of the mutant p53 in both p16<sup>INK4a</sup><sup>+/+</sup> and <sup>/-</sup> cells was documented by Western blotting one passage after infection (data not shown). Although no difference in 48-h growth curves, cell cycle profile, or cdk4 complex composition was seen in p16<sup>INK4a</sup><sup>/</sup><sup>-</sup> versus <sup>+/</sup> cells expressing this mutant form of p53 (DNp53) after infection (data not shown), a 48% increase in cell number was seen in DNp53 cells that were grown but not passaged for 10 days (data not shown). That is, under conditions used for standard growth curve determination, DNp53 cultures accumulated to very high densities, suggesting this cooperation between p16<sup>INK4a</sup> loss and DNp53 might reflect a contribution of p16<sup>INK4a</sup> described previously to density-mediated arrest (7, 10).

To address further this possibility, replicate cultures of early passage MEFs (p16<sup>INK4a</sup><sup>+/</sup> and <sup>/</sup>) with and without DNp53) were plated and refed, but not passaged, for 21 days. Under these conditions, cells were confluent by day 3. Plates were harvested, cells were counted, and cell cycle profiles were measured on the indicated days; the ratios of p16<sup>INK4a</sup><sup>/</sup><sup>-</sup> versus <sup>+/</sup> cells are shown (Fig. 1a). Consistent with a known role for p16<sup>INK4a</sup> in constraining G<sub>1</sub> exit, S phase fraction was higher in p16<sup>INK4a</sup><sup>/</sup><sup>-</sup> than <sup>+/</sup> cells (without DNp53) on days 7 (27.1 versus 19.8%), 11 (8.3 versus 4.3%), and 14 (4.3 versus 2.9%). No difference in cell size (as determined by forward scatter) was discerned between p16<sup>INK4a</sup><sup>/</sup><sup>-</sup> and <sup>+/</sup> cells on any day of culture. This growth difference was of comparable magnitude regardless of DNp53 status (Fig. 1a). Additionally, this growth difference is not solely attributable to accumulation of p16<sup>INK4a</sup> after prolonged culture as p16<sup>INK4a</sup><sup>/</sup><sup>-</sup> MEFs grow with similar kinetics as <sup>+/</sup> cultures up to passage 8 (i.e., 24 days in culture) in a 3T9 assay, where the cells are serially passaged (11). Along these lines, no difference in the growth of p16<sup>INK4a</sup><sup>/</sup><sup>-</sup> versus <sup>+/</sup> MEFs was noted under a variety of other settings, including after exposure to UV light or ionizing radiation, under hypoxic conditions (1% O<sub>2</sub>), in the setting of H-Ras(G12V) expression, and at low seeding density or low serum (data not shown and Refs. 10 and 11). These data demonstrate that p16<sup>INK4a</sup> plays a role in constraining cellular proliferation at high density, even in cells lacking functional p53.

To validate more rigorously the role of p16<sup>INK4a</sup> in density-mediated growth arrest, a 3T9 assay was performed on wild-type MEFs with standard methods except that the cultures were seeded into plates of varying size (i.e., only the culture density was varied; Fig. 1b). In 6-cm dishes, cultures were confluent at all times; in 10-cm dishes, cultures only became confluent by the 3rd day after passaging; in 15-cm dishes, cultures were never confluent. In 10-cm dishes, cells grew considerably better than cells in either 6- or 15-cm dishes. Western blot analysis (Fig. 1c) of these cultures at passages 3, 5, and 7 showed that p19<sup>ARF</sup> accumulation increased more rapidly in cultures passaged at lower density, whereas p16<sup>INK4a</sup> accumulated to slightly higher levels by passage 7 in the highest density cultures. On the other hand, p19<sup>ARF</sup> expression was markedly reduced in cultures grown at highest density relative to those grown at low density (Fig. 1c). Previously, it has been demonstrated that p19<sup>ARF</sup> (or p14<sup>ARF</sup>) is induced by E2F (13), but p19<sup>ARF</sup> accumulated to highest levels in the lowest density cultures (Fig. 1c). Therefore, the increase in p19<sup>ARF</sup> levels seen in less dense cultures could not be solely attributed to increased proliferation, as the least dense cultures (15 cm) did not proliferate as extensively as the intermediate density cultures (10 cm; Fig. 1b). These results suggest that the role of p16<sup>INK4a</sup> in the regulation of...
MEF growth only becomes apparent when it is induced to sufficient levels (e.g., by prolonged growth at high density) and primarily in the setting of low p19ARF levels or compromised p53 function.

**p16INK4a Loss Augments the Transformation of p53-deficient Cells.** These cooperative effects of p16INK4a and p53 loss in the growth of primary cells prompted an examination of their interactions in modulating foci formation, particularly because focus formation in part reflects unrestricted growth at high density. Classical transformation assays were performed on early passage MEFs by transfecting in combination H-RAS(G12V), a dominant-negative mutant form of H-RAS(G12V) in this assay, p53 and H-Ras(G12V) cotransfections; the latter was consistent with the ability of T-Ag to inactivate Rb, thereby rendering p16INK4a status irrelevant. Likewise, p16INK4a loss did not obviate the need for H-Ras(G12V) in this assay, as p16INK4a−/− cells transfected with p53-DD or T-Ag alone did not form foci (data not shown). Transformed foci from H-Ras(G12V) and p53-DD transfections could be subcloned with comparable frequency regardless of p16INK4a genotype (18 of 18 for p16INK4a−/− versus 17 of 18 for p16INK4a+/−). These data demonstrate that the loss of p16INK4a function can enhance the growth and transformation of cells harboring compromised p53 function.

**p53 and p16INK4a Loss Cooperate to Increase the Rate of Tumorigenesis and Expand the Spectrum of Tumor Types.** Given the in vitro cooperation in transformation assay between p16INK4a and p53 loss, we sought to extend these observations by assessing the effects of dual p53 and p16INK4a inactivation on tumorigenesis in vivo. Toward this end, cohorts of animals harboring the p16INK4a−/− allele on the p53+/− or p53−/− backgrounds were monitored (Figs. 2, a–c and Table 1). As shown previously, mice lacking either one or both copies of p16INK4a are more tumor prone than littermate wild-type animals, with p16INK4a−/− mice developing sarcomas of various types, lymphomas, and melanomas with a mean latency of 76 weeks (Fig. 2a and Table 1). Median tumor latency was shortened in the p53+/− (42 weeks) and p53−/− (10 weeks) cohorts (Fig. 2, b and c and Table 1). Regardless of p53 genotype, p16INK4a−/− mice were more tumor prone than p16INK4a+/− mice, with heterozygous animals demonstrating...

**Table 1** Spontaneous tumor spectrum of p16INK4a and p53-deficient mice (see “Materials and Methods” for histologic description and criteria)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tumor Type</th>
<th>Number of Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53+/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16INK4a+/−</td>
<td>(1 tumor from 41 mice)</td>
<td>1 Malignant spindle cell neoplasm</td>
</tr>
<tr>
<td>(15 tumors from 120 mice)</td>
<td>5 Lymphomas (4 histiocytic)</td>
<td></td>
</tr>
<tr>
<td>p16INK4a−/−</td>
<td>(17 tumors from 50 mice)</td>
<td>5 Malignant spindle cell neoplasms</td>
</tr>
<tr>
<td>(42 weeks) and p53+/− (10 weeks) cohorts</td>
<td>2 Angiosarcomas</td>
<td></td>
</tr>
<tr>
<td>(10 weeks)</td>
<td></td>
<td>2 Osteosarcomas</td>
</tr>
<tr>
<td>p53−/−</td>
<td>(2 tumors from 11 mice)</td>
<td>1 Squamous cell carcinoma</td>
</tr>
<tr>
<td>p16INK4a+/−</td>
<td>(5 tumors from 13 mice)</td>
<td>5 Malignant spindle cell neoplasms</td>
</tr>
<tr>
<td>(9 tumors from 11 mice)</td>
<td>4 Angiosarcomas</td>
<td></td>
</tr>
<tr>
<td>p16INK4a−/−</td>
<td>(2 Malignant spindle cell neoplasm)</td>
<td></td>
</tr>
<tr>
<td>p53+/−</td>
<td>(9 tumors from 10 mice)</td>
<td>2 Osteosarcomas</td>
</tr>
<tr>
<td>p16INK4a+/−</td>
<td>(19 tumors from 16 mice)</td>
<td>1 Squamous cell carcinoma</td>
</tr>
<tr>
<td>p53−/−</td>
<td>(23 tumors from 22 mice)</td>
<td>5 Thymic lymphomas (small lymphocytic)</td>
</tr>
<tr>
<td>p16INK4a+/−</td>
<td>(19 tumors from 16 mice)</td>
<td>2 Angiosarcomas (1 metastatic)</td>
</tr>
<tr>
<td>p53+/−</td>
<td>(9 tumors from 10 mice)</td>
<td>2 Malignant spindle cell neoplasms</td>
</tr>
<tr>
<td>p16INK4a+/−</td>
<td>(2 Malignant spindle cell neoplasm)</td>
<td></td>
</tr>
<tr>
<td>p53−/−</td>
<td>(10 Angiosarcomas (2 metastatic)</td>
<td></td>
</tr>
<tr>
<td>p16INK4a+/−</td>
<td>(6 Malignant spindle cell neoplasms)</td>
<td></td>
</tr>
<tr>
<td>p53+/−</td>
<td>(9 Angiosarcoma (5 metastatic)</td>
<td></td>
</tr>
<tr>
<td>p16INK4a+/−</td>
<td>(6 Thymic lymphomas (small lymphocytic)</td>
<td></td>
</tr>
<tr>
<td>p53+/−</td>
<td>(1 LN Lymphoma (small lymphocytic)</td>
<td></td>
</tr>
<tr>
<td>p16INK4a+/−</td>
<td>(1 Malignt spindle cell neoplasm)</td>
<td></td>
</tr>
</tbody>
</table>

---

Fig. 2. p16INK4a and p53 deficiency cooperate in tumorigenesis in vivo. Tumor-free survival curves are shown by p53 and p16INK4a genotype (a, p53+/−; b, p53+/−; c, p53−/−).

Curves are compared with a Log-rank test, and *P* is for the trend of increasing tumor susceptibility with decreasing p16INK4a gene dosage. d. Western blot analysis of spontaneous tumors in p16INK4a+/− and −/− mice. Results from 15 representative tumors are shown by p16INK4a genotype. p16INK4a−/− mice, p53−/−, and tubulin results are obtained by probing of the same membrane; mdm2 results are from a different membrane. c. control (3T3DM cells for mdm2 and UV-irradiated p16INK4a−/− cells for other proteins). e. MSP was performed on p53+/− and −/− tumors with low or undetectable p16INK4a expression (except tumor #15). p16INK4a and p53 genotypes are indicated. Tumor numbers are the same as in Fig. 1d. The presence of an unmethylated band in samples from primary tumors is indicative of stromal contamination.

N, normal lung; S, Sp6, a methylated lung cancer cell line (19); U, DNA that was not bisulfite treated.
Table 2a Mutual inactivation of p16/Rb and p19/p53 in spontaneous tumors

<table>
<thead>
<tr>
<th>p16Ink4a-Rb lesion</th>
<th>p19ARF-mdm2/p53 lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>0/1</td>
</tr>
<tr>
<td>p16Ink4a&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>9/10</td>
</tr>
<tr>
<td>p16Ink4a&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>7/7</td>
</tr>
<tr>
<td>Total</td>
<td>16/18</td>
</tr>
<tr>
<td>p53&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>3/4</td>
</tr>
<tr>
<td>p16Ink4a&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>4/11</td>
</tr>
<tr>
<td>p16Ink4a&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>2/2</td>
</tr>
<tr>
<td>Total</td>
<td>9/17</td>
</tr>
</tbody>
</table>

Table 2b Mechanism of p16 loss by p53 genotype

<table>
<thead>
<tr>
<th>p16 loss</th>
<th>Methylation</th>
<th>LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>17/19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12/27</td>
</tr>
<tr>
<td>p53&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>7/5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3/7</td>
</tr>
<tr>
<td>Total</td>
<td>24/34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15/34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P = 0.01 for p53<sup>+/+</sup> versus p53<sup>-/-</sup> tumors.<br>
<sup>b</sup> P < 0.0001 for loss by methylation versus LOH.

Discussion

The infrequent detection of gene deletion in tumors lacking p16<sup>INK4a</sup> expression suggested the possibility of gene silencing through promoter methylation, which has been noted previously to occur in primary murine tumors (11, 19). Therefore, 17 tumors (10 p53<sup>+/+</sup> and 7 p53<sup>-/-</sup>) were analyzed (Fig. 2e and Table 2b) using an MSP described previously (11, 18). Of 16 tumors without detectable p16<sup>INK4a</sup> expression, 8 showed evidence of promoter methylation in bisulfate-treated DNA from primary tumors. Methylation was not detected in a tumor with deletion of the wild-type p16<sup>INK4a</sup> allele (tumor #13; Fig. 2e) or in a tumor with detectable p16<sup>INK4a</sup> expression (tumor #15). It is worth noting that the PCR strategy will not amplify a product from the null allele (e.g., tumor #8) because the 3′ primer is located in the targeted deletion of exon 1α (11). These data suggest that loss of p16<sup>INK4a</sup> occurs more readily through promoter methylation than deletion (Table 2b), even in the p53<sup>-/-</sup> setting in which the occurrence of chromosome loss and genome-wide interstitial deletions have been purported to be more frequent (17, 20, 21).

The frequencies of p16<sup>INK4a</sup> loss of expression, promoter methylation, and LOH in tumors from this work and a previous analysis (11) are compiled in Table 2b, indicating a significant predilection for methylation over LOH (P < 0.0001), in tumors where p16<sup>INK4a</sup> expression is lost. A caveat to this analysis is that the majority of tumors included in Table 2b were from p16<sup>INK4a</sup>+/− mice (27 of the 34 tumors in the p16<sup>INK4a</sup> loss column), and it is possible that the frequency of LOH would be higher in tumors from p16<sup>INK4a</sup>−/− mice. Additionally, ~50% of tumors with undetectable p16<sup>INK4a</sup> expression did not demonstrate either LOH or methylation, suggesting either that some other mechanism of silencing of p16<sup>INK4a</sup> occurs or that our MSP strategy is unable to detect partial promoter methylation.
tumorigenesis. A few features of these results are notable and somewhat unexpected. In an effort to identify a p53-independent general feature of a growing tumor that induces p16INK4a, we found that p16INK4a limited cellular growth at high density in accord with previous observations (7, 10). Although other potentially tumor-related stimuli (e.g., UV light, ionizing radiation, and Ras expression) have been reported to induce p16INK4a (8, 9, 22, 23), germ-line loss of p16INK4a has only been shown to confer a growth advantage in MEFs when passaged serially for long periods (i.e., in a 3T9 analysis) or at high density (this work and Refs. 10 and 11). This difference in the importance of p16INK4a between these results and those of other groups may reflect cell type and/or interspecies differences, e.g., although p16INK4a deficiency has minimal effects in short-term MEF cultures, p16INK4a inactivation enhances the growth of primary murine T cells and bone marrow-derived macrophages (11, 24), as well as human fibroblasts and keratinocytes (5, 25). The molecular basis for these differences is not currently known. Our data are consistent, however, with the notion that growth at high density (as in an incipient tumor) provides a selection pressure to delete or silence p16INK4a.

Furthermore, although p16INK4a expression varied only modestly in cultures of differing densities (Fig. 1c), p19ARF levels varied tremendously. Previously, several groups have demonstrated p19ARF (or p14ARF) regulation by E2F (13), and the observation of lower levels of p19ARF in cells passaged at highest density likely reflects decreased proliferation. As cultures passaged at intermediate density grew most rapidly (Fig. 1c), some feature of low density besides increased proliferation/ passage number appears to induce p19ARF expression. A possible interpretation of these results is that the two products of the Ink4a/Arf locus are regulated differentially by signals stemming from cell-cell interactions and/or paracrine factors. Elucidating the molecular nature of these density-mediated signals, and determining if they are the same signals that mediate p16INK4a induction and ultimately loss, in nascent tumors may lead to an improved understanding of this barrier to cancer.

Lastly, our data permit an in vivo quantification of the preference of p16INK4a loss through promoter methylation versus gene deletion. Table 2b represents pooled data from this work and a previous analysis of carcinogen-induced tumors (11). These observations suggest that methylation of p16INK4a is ~10-fold more probable than LOH, even in p53−/− mice, where LOH occurs with increased frequency through chromosome loss or interstitial deletion (17, 20, 21). Further support for this observation comes from the fact that p16INK4a methylation as a selection event can be observed in cultured primary murine macrophages or human mammary keratinocytes (24, 25). These in vitro data suggest that p16INK4a induction, in response to culture-related stimuli, limits growth of these cell types. Our in vivo data suggest that corresponding cancer-related stimuli leads to p16INK4a induction in nascent tumors. Selection for clones with methylation-dependent silencing of p16INK4a in either case does not appear to require transformation per se but rather reflects a limit to growth imposed by p16INK4a. Our data are consistent with the high frequency of p16INK4a methylation seen in human cancers and suggests p16INK4a+/− mice provide a useful platform for the study of this process.

Acknowledgments

We thank M. Butler, J. Decaprio, W. Kaelin, T. Devereux, and M. Oren for advice and reagents and N. Bardeesy, M. Ivan, and P. Sicinski for advice and critical reading of the manuscript.

References
p16\textsuperscript{INK4a} and p53 Deficiency Cooperate in Tumorigenesis
Norman E. Sharpless, Scott Alson, Suzanne Chan, et al.


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/10/2761

Cited articles This article cites 23 articles, 13 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/10/2761.full#ref-list-1

Citing articles This article has been cited by 14 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/10/2761.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.