Heterozygosity of \( p21^{WAF1/CIP1} \) Enhances Tumor Cell Proliferation and Cyclin D1-associated Kinase Activity in a Murine Mammary Cancer Model

Jeffrey M. Jones, Xian-Shu Cui, Daniel Medina, and Lawrence A. Donehower

Division of Molecular Virology [J. M. J., X.-S. C., L. A. D.] and Department of Cell Biology [D. M., L. A. D.], Baylor College of Medicine, Houston, Texas 77030

Abstract
The \( p21^{WAF1/CIP1} \) cyclin-dependent kinase (cdk) inhibitor is a regulator of the G1-S cell cycle checkpoint. Despite the importance of \( p21 \) in cell cycle inhibition, its role as a tumor suppressor is uncertain. \( p21 \) mutations are infrequent in human tumors, and null mice exhibit no increased tumor incidence. To ascertain whether \( p21 \) could influence tumor formation or progression in the context of other oncogenic stimuli, we crossed \( p21 \)-deficient mice with mammary tumor susceptible \( Wnt-1 \) transgenic mice. The \( p21^{+/+} \), \( p21^{++/-} \), and \( p21^{-/-} \) \( Wnt-1 \) transgenic female offspring were monitored for mammary tumor incidence and growth rates. \( p21 \) status had no effect on the age at which mammary tumors formed. However, \( p21^{+/+} \) mammary tumors grew significantly faster than \( p21^{++/-} \) and \( p21^{-/-} \) mammary tumors. The increased growth rates were confirmed by mitotic index counts and by BrdUrd labelling assays, indicating that a significantly higher percentage of \( p21^{+/+} \) tumor cells were in S phase and mitosis than their \( p21^{++/-} \) and \( p21^{-/-} \) counterparts. Moreover, cyclin D1-associated phosphorylation of retinoblastoma protein was significantly increased in \( p21^{+/+} \) tumor lysates compared with \( p21^{++/-} \) and \( p21^{-/-} \) lysates. These results are consistent with data indicating that reduced levels of \( p21 \) can facilitate cyclin/cdk complex formation while enhancing cdk activity. Thus, a reduction of \( p21 \) dosage may promote tumor progression in the presence of other oncogenic initiators. The dependence of \( p21 \) on prior oncogenic stimuli for its tumor-promoting activities suggests that it may behave as a tumor modifier gene rather than as a tumor suppressor gene.

Introduction
The cell cycle is characterized by a series of ordered steps leading to the precise duplication of the genetic complement of the cell and the segregation of an identical set of duplicated chromosomes to each of the two daughter cells. This process is tightly regulated through a series of cell cycle checkpoints that serve to regulate the progression from one stage of the cell cycle to the next, ensuring that each step has been completed without error before entry to the next stage (1, 2). When this process becomes deregulated it can lead to aberrant cell growth, hyperproliferation, and, eventually, cancer (3, 4). The progression through these checkpoints is mediated by the cyclins, cdks, and the cdk inhibitors.

Progression through the G1-S checkpoint requires phosphorylation of the Rb protein by both cyclin D and E complexes (5). The phosphorylation of Rb disrupts its binding to members of the E2F transcription factor family (6). E2F release is necessary for the transcription of a variety of S phase genes (7). One of the primary negative regulators of the Rb phosphorylation event is the \( p21^{WAF1/CIP1} \) cdk inhibitor (8, 9). \( p21 \) functions by binding to and inactivating a number of the cyclin/cdk complexes involved in the G1-S transition (8–10). By preventing the cdks from phosphorylating their targets, the cell cycle is halted and the checkpoint is activated. \( p21 \) has been shown in a number of studies to be an important component of both p53-dependent (10–12) and -independent (13–15) cell cycle checkpoint pathways. Recently, \( p21 \) has been shown to inhibit G2-M phase transition, as well as the G1-S transition (16–19). Thus, \( p21 \) plays a critical role in regulating cell cycle progression and protecting cells against DNA damage and other oncogenic hazards.

It has been observed that cyclin/cdk complexes can be present in both active and inactive forms (20) and that they must be active to allow progression through the cell cycle. Multiple groups have described the active cyclin/cdk complex as a quaternary structure containing, in addition to the cyclin and cdk, a subunit of proliferating cell nuclear antigen, and, in the case of G1-S-related complexes, \( p21 \) (10, 20, 21).

Received 1/11/99; revised 3/3/99; accepted 3/11/99.

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.

1Supported by Department of Defense Breast Cancer Grant DAMD17-94-J-4401 and National Cancer Institute Grant CA54897. L. A. D. is the recipient of a Research Career Development Award from the National Cancer Institute, NIH.

2To whom requests for reprints should be addressed, at Division of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-3594; Fax: (713) 798-3490; E-mail: larryd@bcm.tmc.edu.

3The abbreviations used are: cdk, cyclin-dependent kinase; Rb, retinoblastoma; BrdUrd, bromodeoxyuridine; LOH, loss of heterozygosity; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; HPF, high-powered field; ATM, ataxia telangiectasia; MMTV, mouse mammary tumor virus.
p21 as a Tumor Modifier Gene

Although p21 null mice are developmentally normal, they partially affected it (26–28). However, p21 null embryo fibroblasts showed an enhanced growth rate in cell culture and were deficient in G₁ arrest after exposure to DNA damage and nucleotide pool perturbation (29, 30). Although these results confirmed the importance of p21 in mediating G₁ checkpoint control, they failed to establish p21 as an important antioncogenetic molecule. However, subsequent to these experiments, Missero et al. (31) showed that keratinocytes derived from p21 null animals transformed with activated ras demonstrated a significantly higher tumorigenic capacity compared with their ras-transformed p21+/+ counterparts. This result suggested that in the presence of other oncogenic stimuli (e.g., activated ras), p21 deficiency might increase the aggressiveness of tumor cells. On the other hand, in the context of ATM-deficiency, the absence of p21 actually delayed the thymic lymphomagenesis normally observed in ATM null mice (32). This latter effect may be a result of a sensitized apoptotic response associated with loss of p21, which has been observed in other cell systems as well (8, 33, 34).

To further address the effects of p21 on aspects of tumor initiation and progression, we have generated a mouse model in which mammary tumors arise in the presence of varying dosages of p21. This has been accomplished by crossing p21-deficient mice (30) with mammary tumor-susceptible Wnt-1 transgenic mice (35). The Wnt-1 transgenic mice carry a Wnt-1 oncogene, the expression of which is driven by a mammary gland-specific MMTV promoter. All Wnt-1 female transgenic animals develop mammary adenocarcinomas at an early age (35). Crossing of the Wnt-1 transgenic mice with p21-deficient mice resulted in female Wnt-1

Fig. 1. Tumor incidence in female Wnt-1 transgenic mice of different p21 genotypes. Animals were monitored for tumors on a weekly basis by both observation and palpation. Ages in weeks were recorded at the time of first tumor observation. There were no significant p21-dependent differences in tumor incidence in the mice. The average age at first tumor observation was 23 weeks for p21+/+ (n = 16), p21+/− (n = 20), and p21−−/− (n = 9) female Wnt-1 transgenic mice.

Fig. 2. p21-dependent differences in growth rates of mammary tumors arising in female Wnt-1 transgenic mice. After the first observation of a mammary tumor, tumor size was measured weekly thereafter with calipers, and tumor volumes were calculated as previously described by Miller et al. (31). Time 1 represents the first date at which the tumor was observed, and Times 2–4 represent weekly subsequent measures of tumor volume. Average tumor volumes for each p21 genotype at weekly intervals are indicated by the appropriately shaded bars. Bars representing SE determinations are indicated for p21+/+ tumors (n = 15), p21+/− tumors (n = 17), and p21−−/− tumors (n = 9).
that all p21 mutant one tumor had deleted the mutant p21 allele migrating at 9 kb from the wild-type allele status in Wnt-1 transgenic p21+/− mammary tumors. Tumor DNA from p21+/− tumors was isolated, cleaved with Bgl II, and subjected to Southern blot hybridization with a murine p21 probe that distinguishes the mutant p21 allele migrating at 9 kb from the wild-type p21 allele migrating at 8 kb (25). Note that all p21+/− tumors retained an intact wild-type p21 allele, although one tumor had deleted the mutant p21 allele (arrow).

transgenic offspring with zero (p21−/−), one (p21+/−), or two (p21+/+) wild-type p21 alleles. We discovered that although varying p21 levels had no effect on mammary tumor incidence, the actual growth rate of the tumors was affected by p21 dosage. Surprisingly, p21+/− mammary tumors grew dramatically faster than either p21+/+ or p21−/− mammary tumors. This effect of p21 heterozygosity on increased tumor growth rate was corroborated at the cellular and molecular level by tumor cell proliferation assays and assays for cyclin D1-associated kinase activity. The data presented here shows that in certain in vivo contexts, reduced p21 dosage can promote tumor progression. Moreover, we show that low to moderate levels of p21, but not its absence, may have growth stimulatory effects in some tumor types.

Results
Effects of p21 Dosage on Tumor Incidence, Pathology, and Growth Rate. To assess the effects of p21 dosage in a tumorigenic context, we crossed p21-deficient mice (30) with mammary tumor-susceptible Wnt-1 transgenic mice (35). Wnt-1 transgenic female offspring of all three p21 genotypes (p21+/+, p21+/−, and p21−/−) were monitored on a weekly basis for tumor incidence and tumor growth rates. As shown in Fig. 1, the p21 genotype had no significant effect on the age at which the animals developed tumors. All Wnt-1 transgenic females, regardless of p21 genotype, developed mammary tumors at a mean age of 23 weeks, and all had died of mammary tumors by the age of 45 weeks.

The general morphology of the Wnt-1 transgenic tumors was unchanged by p21 status. Tumors in all groups of mice were predominantly of Dunn types A and B (36, 37). Type A tumors are composed of microacini lined by a single layer of cuboidal epithelium, whereas type B tumors show a variable histology with both well-differentiated and poorly differentiated regions of cells arranged in irregular cords, sheets, or balls. The cytoplasm of the tumor cells is slightly basophilic. This appearance is in marked contrast to mammary tumors induced by specific oncogenes in transgenic mice, where the tumor phenotype is dictated by the transgene. For instance, activated ras-induced mammary tumors have a papillary appearance and are composed of a relatively homogeneous population of cells with a dense eosinophilic cytoplasm (38, 39). The results of our studies support the hypothesis of Cardiff and Wellings (38, 39) that specific transgenes determine the tumor histopathology.

In addition to tumor incidence rates, we monitored tumor growth rates. Mice were checked weekly for tumors by palpation. From the time at which a tumor was first observed it was measured with calipers in two dimensions and its volume was determined. Tumor volume was then measured on a weekly basis for 4 weeks. As indicated in Fig. 2, p21+/+ tumors grew at the slowest rate. p21−/− tumors grew only slightly faster, whereas p21+/− tumors expanded significantly faster than either p21+/+ or p21−/− tumors. p21+/− tumors grew at a rate similar to those tumors that have been observed in p53−/− Wnt-1 transgenic mice we have described previously (40).

Wild-type p21 Allele Status in p21+/− Tumors. The rapid growth rate of the p21+/− tumors might be due, in part, to selective loss of the remaining wild-type p21 allele. Such wild-type allele loss occurs frequently in p53−/− tumors and results in accelerated tumor growth rates (40, 41). To assess p21 allele status in the p21+/− tumors, we performed Southern blot hybridization analysis on DNA from 17 p21+/− tumors using a murine p21 probe (Fig. 3). The results indicated that all p21+/− tumors retained an intact p21 allele. In fact, one tumor exhibited loss of the mutant p21 allele, suggesting that there may be a selective advantage in retaining a single wild-type p21 allele during tumorigenesis.

Tumor Cell Proliferation Rates Are Highest in p21+/− Mammary Tumors. Given the role of p21 in cell cycle regulation, we hypothesized that the observed p21-dependent tumor growth rate differences were due to changes in cell proliferation rates. To assess tumor cell proliferation, we used two assays, mitotic figure counts, and BrdUrd incor-
poration assays. The mitotic figure assay provides an estimate of the percentage of cells in a tumor undergoing mitosis at one particular time. Mitotic figure counts were performed on H&E-stained sections from 16 p21<sup>+/1</sup>, 18 p21<sup>+/2</sup>, and 11 p21<sup>−/−</sup> tumors. p21<sup>+/+</sup> tumors had on average 45.4 mitotic figures/10 HPFs, compared with 58.5 for the p21<sup>+/−</sup> and 45.3 for the p21<sup>−/−</sup>, as shown in Fig. 4. Statistical analysis of these results indicate a significant increase in mitotic levels in p21<sup>+/2</sup> tumors compared with either p21<sup>+/1</sup> (\(P = 0.0076\)) or p21<sup>−/2</sup> tumors (\(P = 0.016\)).
To further confirm the increased proliferation levels observed in p21+/− tumors, BrdUrd incorporation assays were performed. The BrdUrd incorporation assay assessed the percentage of tumor cells that were actively replicating their DNA over a 3-h labeling period. Tumor-bearing animals were given injections of BrdUrd 3 h before sacrifice. After sacrifice, fixed tumor sections were prepared and subjected to standard immunohistochemical detection methods using an anti-BrdUrd antibody (Fig. 5, A–C). p21+/− tumors exhibited on average 10.9% of cells labeling (n = 13), whereas the p21+/+/ and p21−−/− tumors exhibited 7.4% (n = 15) and 5.8% (n = 9), respectively (Fig. 5D). Statistical analyses indicated that p21+/− tumors exhibited significantly higher levels of cells in S phase than either p21+/+/ tumors (P = 0.01) or p21−−/− tumors (P = 0.004). The results of this assay are consistent with the results of the mitotic figure counts in showing an increase in cell proliferation levels in the p21+/− tumors compared with the p21+/+/ and p21−−/− tumors.

Finally, to determine whether the increased proliferation of the p21+/− tumor cells was a late event during tumor progression or actually preceded tumor formation, we examined BrdUrd incorporation in the hyperplastic mammary glands of the Wnt-1 transgenic female mice of each p21 genotype. Because the Wnt-1 transgenic mammary glands are immediately hyperplastic after initial formation (35), it was impossible to obtain normal nonhyperplastic mammary glands. As for the mammary tumors, BrdUrd incorporation was monitored in the mammary gland cells 3 h after injection with BrdUrd. Interestingly, although there was variability from tumor to tumor, on average p21+/− mammary gland cells showed roughly a 2-fold increase in the fraction of cells with incorporated BrdUrd compared with p21+/+/ and p21−−/− mammary glands (Fig. 5E). The proliferation differences were statistically significant for the p21+/− to p21+/+/ comparison and near statistical significance for the p21+/− to p21−−/− comparison. However, the absolute fraction of mammary gland cells exhibiting incorporation was, on average, roughly 10-fold less than that observed in mammary tumor cells.

**Tumor Cell Apoptosis Levels Are Unaffected by p21 Status.** Tumor growth rates can be affected by both rates of cell proliferation and cell death. Because the absence of p21 has been shown to sensitize cells to apoptosis in other model systems (33, 34, 42), it was important to determine whether the observed differences in tumor growth rates were due largely to alterations in rates of cell proliferation rather than cell death. To determine apoptotic levels in the p21+/Wnt-1 tumor samples, they were stained immunohistochemically by TUNEL assay. As shown in Fig. 6, the levels of apoptosis in the mammary tumors were very low, typically around 1%, and there was no p21−−/− dependent variation in tumor cell apoptotic levels.

**Increased Cyclin D1-associated Rb Kinase Activity in p21+/− Tumors.** We hypothesized that the increased proliferation observed in the p21+/− tumors could be a function of reduced levels of p21 protein associated with the G1 cyclin/cdk complexes. This reduced p21 association might result in increased phosphorylation of Rb and an increased likelihood of entry into S phase in the p21+/− tumor cells. Immunoprecipitation-Western blot assays indicated that, on average, reduced levels of p21 were associated with cyclin D1/cdk complexes in the p21+/− tumor cells compared with p21+/+ tumor cells, but the tumor to tumor variability precluded demonstration of statistically significant differences (data not shown). Nevertheless, reduced levels of p21 in p21+/− tumor cells would be consistent with higher levels of intrinsic kinase activity of G1 cyclin/cdk complexes than in p21+/+/ tumor cells. To test this, we immunoprecipitated the lysates from p21+/+, p21+/−, and p21−−/− tumors with an antibody to cyclin D1. The immunoprecipitated cyclin D1 complexes were then used to phosphorylate a Rb substrate. Complexes immunoprecipitated from p21+/−/− tumors exhibited, on average, at least 2-fold higher activity than complexes prepared from either p21+/+ or p21−−/− tumors (Fig. 7). Although there was considerable variability among the tumors, the mean Rb kinase activity averaged from 10 tumors of each p21 genotype show increases in the p21+/− tumors. Control reactions without Rb substrate or immunoprecipitations with antibodies to noncyclin/cdk proteins failed to generate phosphorylated bands of the appropriate size (data not shown).

The heterogeneity observed in the levels of Rb kinase activity among the various tumors mirrored to some extent the heterogeneity of tumor growth rates. To determine whether there was a close correlation between tumor growth rates and cyclin D1-associated Rb kinase activity, we plotted the final tumor size at harvest for each sample (as an indicator of tumor growth rate) and overlaid this with a graph of the individual tumor Rb kinase levels (Fig. 8). The results show a very close correlation between tumor growth rates and their respective Rb kinase activities, suggesting that the observed tumor growth rates were dependent, at least in part, on the activity of the cyclin D1-associated cdks.

**Discussion**

The evidence that p21 is a tumor suppressor has been mixed. On the one hand, introduction of exogenous p21 into tumor cells can often suppress their growth (11, 23, 24). On the other hand, the failure to detect tumors in p21-deficient mice and the relative infrequency of p21 mutations in human tumors indicate that p21 dysfunction is unlikely to promote tumor formation by itself. In vivo data from this study and other studies (31, 32) all indicate that p21 deficiency can affect tumor progression in the context of other oncogenic stimuli in various ways. Missero et al. (31) demonstrated that p21−−/− keratinocytes infected with an activated ras-containing retrovirus formed tumors more frequently than p21+/+ cells transformed with the same retrovirus. Moreover, the p21−−/− tumors grew much larger and had a much less differentiated histological appearance compared with their p21+/+ counterparts. Bearss et al. (4) have also demonstrated that MMTV-ras transgenic mice which are null for p21 show accelerated tumor incidence and growth rates compared with MMTV-ras transgenic mice with wild-type p21.

---

4 D. J. Bearss, R. Lee, D. Troyer, R. Pestell, and J. J. Windle. p21WAF1/CIP1 deficiency has opposite effects on tumor cell proliferation in mammary tumors from MMTV-ras and MMTV-myc mice, submitted for publication.
This same group has also shown that mammary tumor-susceptible MMTV-c-myc mice exhibit retarded tumor development in the absence of p21. Moreover, Wang et al. (32) have shown that thymic lymphomagenesis induced by ATM deficiency is delayed by loss of p21. Thus, in at least two other in vivo tumorigenesis contexts, reduction of p21 dosage stimulates oncogenic progression, while in other cases, absence of p21 seems to retard tumor progression.

These models and our own p21 model clearly show that in some tumor types, p21 deficiency can influence tumor formation and/or progression in the presence of other initiating oncogenes. Our model differs from the others in that it is p21 heterozygosity rather than p21 nullizygosity, which affects tumor progression. Because both our model and those of Bearss et al. (4) are mammary gland models, the basis for this difference is likely to be based on the initiating oncogene rather than the target cell type. In the case of the ras models, recent studies have shown that activated ras induces p21 and arrests cell growth (43, 44). Only signalling by the Ras-related GTPase Rho could suppress p21 induction (43). Thus, in the absence of p21, the ras tumor models would have no effective downstream block to the effects of acti-
associated complexes were then incubated with Rb substrate protein and lysates were immunoprecipitated with cyclin D1 antibody. Cyclin D1- significantly higher Rb kinase activity than do p21 presented as a bar graph with bars. 

Fig. 7. Rb kinase activity in cyclin D1-associated complexes from Wnt-1 transgenic p21+/+ and p21−−−, and p21+/− and p21−−− mammmary tumors. Tumor lysates were immunoprecipitated with cyclin D1 antibody. Cyclin D1-associated complexes were then incubated with Rb substrate protein and γ-32P-ATP in a kinase reaction before SDS-PAGE and autoradiography, A, phosphorylated Rb is shown for five tumors of each p21 genotype. B, compilation of mean levels of cyclin D1-associated Rb phosphorylation in p21+/+, p21+/−, and p21−−− tumors. A Molecular Dynamics Storm 860 Phosphorimager was used to quantitate intensities of the Rb bands shown in A, and these values were averaged for each p21 genotype and presented as a bar graph with bars. Note that the p21+/− tumors have significantly higher Rb kinase activity than do p21+/+ and p21−−− tumors.

mediated Ras signalling, and both tumor initiation and progression are augmented.

In our model, Wnt-1 overexpression probably does not induce Ras signalling pathways to the same extent. Thus, tumor incidences are not affected by reduction or loss of p21. Rather, tumor growth rates are affected in a p21-dosage-dependent manner. However, the accelerated growth rates of the p21 heterozygous tumor cells, in comparison with wild type and null p21 tumor cells, was surprising. Moreover, this differential proliferation effect seems to precede actual mammary tumor formation because it is also observed in the hyperplastic mammary gland cells. Thus, p21 heterozygosity in this model does not seem to affect tumor initiation, but does affect tumor progression through increased tumor growth rates.

The phenomenon of increased tumor cell proliferation in the p21+/− tumors is consistent with the concentration-dependent effects of p21 demonstrated by Zhang et al. (20) and LaBaer et al. (21). Although high levels of p21 inhibited cyclin/cdk kinase activity, moderate levels of p21 actually had a kinase-stimulatory effect. However, very low levels of p21 were also inhibitory to cyclin/cdk kinase activity. LaBaer et al. (21) demonstrated that p21 directly mediated cyclin/cdk complex formation and enhanced the affinity of the two molecules by 35-fold. Thus, it was proposed that low to moderate levels of p21 facilitated G1 cyclin/cdk assembly without inhibiting the intrinsic kinase activity of the complex. However, with increasing p21 concentrations, cyclin/cdk activities dramatically declined, implying that more than one p21 molecule could bind to the complex and these additional molecules were inhibitory rather than stimulatory (20, 21).

Another interpretation is derived from the recent data of Hengst et al. (22), who demonstrated that even one molecule of p21 is inhibitory to the cyclin/cdk complex. The apparent stimulatory effect of low levels of p21 may be due to the fact that the affinity of cdkks and their corresponding cyclins is higher than that of these complexes for p21. Thus, facilitation of the assembly of cyclins and cdkks by p21 may generate a stable complex, but the dissociation of p21 from the complex may be relatively rapid in cells with lower concentrations of p21 (e.g., the p21+/− cells). In such cells, the cdkk activity would be increased compared with cells with a normal complement of p21.

The results we have observed in our in vivo tumor model are consistent with either of the above interpretations. In the p21−−− tumor cells, no p21 is available as an adaptor for assembly of the G1 cyclin/cdk complexes, and these tumors exhibit a slower growth rate. The p21+/− tumors, with only half the normal p21 dosage, may have sufficient quantities of p21 to facilitate assembly of the cyclin/cdk complexes, but insufficient quantities to effectively inactivate all of the complexes. This may be due either to a stoichiometric effect of fewer p21 molecules binding to each cyclin/cdk complex or to a larger fraction of cyclin/cdk complexes which lose their p21 inhibitor and become activated. Although we only assayed cyclin D1-associated cdkk complexes in our model, it is possible that other cyclin complexes, such as cyclin E/cdk2, may also be affected in a similar manner by p21 stoichiometry.
The dependence of p21 on other oncogenic events to exhibit its effects on tumor growth places it more in the category of a modifier gene than a tumor suppressor gene. Modifier genes, as exemplified by the Mom-1 gene affecting intestinal tumor rates in Apc-deficient mice (45), can affect tumor formation and progression only when another tumor-initiating mutation (e.g., an Apc mutation) has occurred (46). Independent of such initiating mutations, a modifier has no apparent tumor initiating or promoting effect. Given the modification of tumor progression observed after reduction of p21 dosage in at least five different mouse tumor model systems (31, 32), and the absence of tumors in p21+/− mice, we propose that p21 may be a tumor modifier gene rather than a tumor suppressor gene.  

The observation that p21 heterozygosity has significant tumor-promoting effects in a murine model may have implications for human tumors. Although assays for specific p21 mutations have revealed that they are relatively infrequent, these same assays have usually not addressed whether the loss of a single p21 allele could play a role in tumor progression. The chromosomal position of p21, 6p21.2 (47), is located in a region that shows LOH in human tumors (48, 49). If p21 heterozygosity is sufficient to promote tumor growth, then mere 6p LOH in a human tumor may confer a tumor growth advantage without requirement for a concomitant mutation in the remaining p21 allele (as expected for a classical tumor suppressor). It will be of interest to determine whether human tumors with 6p LOH have more aggressive growth characteristics on average compared with tumors without 6p LOH.

Materials and Methods

Mice. The generation, biological characterization, and tumor susceptibility of the Wnt-1 transgenic and p21-deficient parental mice used in these studies has been described previously (30, 35). The Wnt-1 transgenic mice were of mixed C57BL/6 and SJL background, whereas the p21-deficient mice were of mixed 129/Sv and NIH Swiss black background. Wnt-1 transgenic males were initially mated to p21+/− females. Wnt-1 transgenic p21−/− male offspring from these crosses were crossed with p21+/− females to obtain Wnt-1 transgenic p21−/− mice. These p21−/− offspring were weaned, genotyped, and monitored weekly for mammary tumors by observation and palpation. When mammary tumors were observed, their diameter was measured in two dimensions by calipers and measured again at 1 week-intervals for 4 weeks before sacrificing the animal and excising the tumor. Part of each tumor was frozen at −80°C for future molecular studies, and the remainder was put in Methacarn fixative for future histopathology.

DNA Isolation and Analysis. The genotypes of offspring resulting from the crosses of the p21-deficient and the Wnt-1 transgenic animals were determined by isolating tail or tumor DNA and performing Southern blot hybridization analysis with Wnt-1- and p21-labelled DNA probes, as described previously (30, 41).

Histological Sample Preparation. Tumors were surgically removed after 4 weeks of growth, and cross-sections were cut with a sterile razor blade. These samples were then fixed in 10% buffered formalin for 4 h at room temperature, embedded in paraffin using standard methods, and sectioned at 5 μm. Sections were then fixed in acetone, and then stored in 95% ethanol until embedding. Samples were then dehydrated with xylenes and embedded in paraffin. Tumor cross-sections were cut with a sterile razor blade. These samples were then fixed overnight in Methacarn, followed by 4 weeks of growth, and cross-sections were cut with a sterile razor blade. These samples were then fixed in acetone, and then stored in 95% ethanol until embedding. Samples were then dehydrated with xylenes and embedded in paraffin. Tumor sections were cut with a microtome to a width of 4 μm and fixed on microscope slides. Some slides were stained with H&E according to standard methods, and other slides were left unstained.

Detection of Apoptotic Cells in Mammary Tumor Samples. Unstained mammary tumor sections were prepared as described above.

Cells undergoing apoptosis were detected using the TACS 2 TdT In Situ Apoptosis Detection Kit ( Trevigen, Gaithersburg, MD). Samples were deparaffinized and treated with Proteinase K (2 μg/ml) for 15 min. Endogenous peroxidase was quenched by immersing samples for 5 min in 3% H2O2. Samples were then incubated with TdT and biotinylated nucleotides for 1 h at 37°C. Cells that had incorporated the biotinylated nucleotide were detected by incubation with streptavidin-horseradish peroxidase, followed by the substrate DAB for 10 min. Samples were then counterstained with 1% Methyl Green for 5 min. Finally, samples were washed sequentially with butanol, ethanol, and xylene and mounted with Permount. Brown-stained cells undergoing apoptosis were then counted in 10 random HPFs within each tumor and averaged with respect to the total cells in each field to give a value of mean percentage of apoptotic cells in each tumor.

Mitotic Figure Determinations. A determination of the number of mitotic figures in tumor samples was performed by counting the number of visible mitotic figures within cells in 10 random HPFs (×400). These fields were all within three HPFs of the edge of standard H&E-stained tumor cross-sections. The total number of mitotic figures was determined for all 10 HPFs, as well as the percentage of tumor cells in the field with visible mitotic figures. All mitotic counts were performed blinded with respect to the p21 genotype.

Brdu Incorporation Assay. BrdU incorporation assays were performed immunohistologically using a cell proliferation kit (RPN 20 Amer sham). Animals were given i.p. injections of 2 ml of BrdUrd labeling reagent/50 g of body weight. DNA labeling was allowed to proceed for 3 h before tumors were harvested. Tumors were then fixed in Methacarn, as described above, and embedded in paraffin. BrdUrd staining was performed in the following manner. Samples were deparaffinized, rehydrated with PBS, and treated with 3% H2O2 to quench endogenous peroxidase. Samples were then incubated with a nuclease/anti-BrdUrd antibody for approximately 1 h after which they were washed with PBS-Tween and PBS. Samples were then incubated for 30 min with a peroxidase-coupled anti-IgG2a antibody (Amersham). Cells that had incorporated the anti-BrdUrd antibody were then visualized via a DAB reaction, which was allowed to proceed for 10 min. Samples were then dehydrated with successive washes of butanol, ethanol, and xylene, followed by mounting with Permount. Black-stained cells indicating BrdUrd incorporation were visualized and counted in 10 random HPFs and averaged for each tumor as a percentage of cells in S phase.

Immunoprecipitation-Western Blot Analyses. Tumor lysate (1.5 mg) was precleared with 15 μg each of protein A and G agarose (Boehringer Mannheim) for 8 h at 4°C. Samples were spun and removed from the protein A and G agarose. Anti-cyclin D1 antibody (5 μg; C-20; Santa Cruz Biotechnology) was added and allowed to bind at 4°C for 8 h. The complex was collected using magnetic beads (Promega) and washed two times with 1X lysis buffer and 50 μl HEPES (pH 7.5). Sample buffer was added, and samples were boiled for 5 min. Samples were run on a 15% SDS-polyacrylamide gel. p21 protein was detected with a mouse IgG monoclonal antibody to p21 (AB-2; Oncogene Research) for 12 h. Blots were washed with Tris-buffered saline Tween, followed by a 4-h incubation with goat antimouse peroxidase (Boehringer Mannheim). Protein was detected using the Supersignal ECL System (Pierce Chemical Co.). Quantiﬁcation of band intensities was performed by densitometry with a Molecular Dynamics Personal Densitometer SI.

Rb Kinase Assay. The cyclin D1-associated complexes were isolated, as described above, except that 700 μg of tumor lysate was used for immunoprecipitation. After the overnight collection with magnetic beads, samples were washed four times with kinase buffer, as described previously (50). The kinase reaction was set up with 48 μl of kinase buffer, 1 μl of Rb substrate (Santa Cruz Biotechnology), and 1 μl of γ-32P-ATP. Samples were incubated for 30 min at 30°C. Incubation sample buffer was added, and samples were boiled for 5 min before running on an 8% polyacrylamide gel. Gels were dried and exposed to film. Kinase levels were quantitated using a Storm 860 Phosphorimager and ImageQuant data analysis software (Molecular Dynamics).


