p18\(^{\text{Ink4c}}\) and \(p53\) Act as Tumor Suppressors in Cyclin D1–Driven Primitive Neuroectodermal Tumor

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Abstract

The retinoblastoma (RB) tumor suppressor pathway is likely important in primitive neuroectodermal tumors (PNET) of the brain. In fact, 10% to 15% of children born with RB mutations develop brain PNETs, commonly in the pineal gland. Cyclin D1, which in association with cyclin-dependent kinase (Cdk) 4 and Cdk6 phosphorylates and inactivates the RB protein, is expressed in 40% of sporadic medulloblastoma, a PNET of the cerebellum. To understand tumorigenic events cooperating with RB pathway disruption in brain PNET, we generated a transgenic mouse where cyclin D1 was expressed in pineal cells. Cyclin D1 enhanced pinealocyte proliferation, causing pineal gland enlargement. However, proliferation ceased beyond 2 weeks of age with reversal of Cdk4-mediated RB phosphorylation despite continued expression of the gene, and the pineal cells showed heterochromatin foci suggestive of a senescent-like state. In the absence of the \(p53\) tumor suppressor, cell proliferation continued, resulting in pineal PNET that limited mouse survival to ~4 months. Interestingly, the Cdk inhibitor p18\(^{\text{Ink4c}}\) was induced in the transgenic pineal glands independently of \(p53\), and transgenic mice that lacked Ink4c developed invasive PNET, albeit at an older age than those lacking \(p53\). Analogous to our mouse model, we found that children with heritable RB often had asymptomatic pineal gland enlargement that only rarely progressed to PNET. Our finding that the Cdk4 inhibitor p18\(^{\text{Ink4c}}\) is a tumor suppressor in cyclin D1–driven PNET suggests that pharmacologic interventions to inhibit Cdk4 activity may be a useful chemoprevention or therapeutic strategy in cancer driven by primary RB pathway disruption.

Introduction

As a tumor suppressor, the retinoblastoma (RB) protein primarily controls entry into S phase of the cell cycle (1). D-type cyclins and their catalytic partners cyclin-dependent kinase (Cdk) 4 and Cdk6 phosphorylate RB to foster the G1 to S phase transition. As might be expected, cyclin D1 is highly expressed and its gene is amplified in a variety of human cancers (reviewed in refs. 2, 3).

Somewhat paradoxically, though, several mouse models have shown cyclin D1 to be rather weakly oncogenic, promoting cancer only after long latency or cooperatively with other genetic changes (4–7). Understanding the secondary genetic changes will provide important insight into the biology of cancer driven by primary RB pathway inactivation.

We focused our studies on primitive neuroectodermal tumors (PNET) originating in the pineal gland of the brain (8). It is clear that the RB tumor suppressor pathway is important in their biology because 10% to 15% of children with heritable RB develop brain PNETs, most commonly originating from the pineal gland. Deregulated cyclin D1 is detectable in over 40% of sporadic infratentorial PNET (called medulloblastoma; ref. 9). Furthermore, medulloblastoma is often driven by deregulation of the Sonic hedgehog (Shh) pathway (10); cyclin D1 is induced by Shh signaling (11) and its absence strongly impedes Shh-driven medulloblastoma (12). We used a transgenic mouse in which human cyclin D1 is driven by the interphotoreceptor retinoid binding protein (Irbp) promoter, which is selectively expressed in the retina and pineal gland (13). Previous studies showed that ectopic cyclin D1 fosters excess proliferation in developing photoreceptor cells, altering retina development, but it does not cause RB (14).

We used the Irbp-cyclin D1 transgenic mouse to explore the tumorigenic effects of primary deregulation of the RB pathway in the pineal gland. We focused our studies on potential interactions between this oncogene and two tumor-suppressive mechanisms involving \((a)\) \(p53\) and a key regulator and effector of its function and \((b)\) p18\(^{\text{Ink4c}}\), which primarily acts to control cyclin D–associated kinase activity. Further, we investigated whether the model we developed was pertinent to pineal gland changes in children with heritable RB.

Materials and Methods

Mouse studies. Two different lines of Irbp-cyclin D1 transgenic mice with indistinguishable phenotypes were used (14). Transgenic mice were bred with \(p53^{−/−}\) (The Jackson Laboratory), \(p21^{−/−}\) (The Jackson Laboratory), Arf\(^{−/−}\)/Cott\(^{−/−}\) (15), or Ink4c\(^{−/−}\) mice (16) and maintained in a mixed C57BL/6 × 129/Sv genetic background. PCR for relevant targeted alleles was used to verify mouse genotypes as previously described (14, 16). Animals were examined at least twice weekly and euthanized at defined experimental time points or when obviously ill in accordance with St. Jude Children's Research Hospital (SJCRH) Animal Care and Use Committee guidelines; all studies were approved by this committee.

Analyses of protein expression in the pineal gland. Protein extracts were prepared from pineal tissue by lysis in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors (50 mmol/L NaF, 20 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 5 mmol/L NaPPO\(_4\), 1 mmol/L phenylmethylsulfonyl fluoride,
1 mmol/L DTT, 10 μg/mL leupeptin, 1 μg/mL pepstatin, 10 μg/mL aprotinin), sonication, and centrifugation. Electrophoresis was performed using NuPage 4% to 12% Bis-Tris or 3% to 8% Tris-acetate gradient gels. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories) and detected using antibodies to p21<sup>Waf1</sup>, Cdk2, Hsc70 (Santa Cruz Biotechnology), p18<sup>Ink4c</sup> (Zymed), and the hemagglutinin (HA) epitope (Covance). RB was immunoprecipitated with anti-RB antibody G3-245 (BD Pharmingen) and detected using either the same antibody or the anti-phospho-Ser<sup>780</sup> (corresponding to Ser<sup>773</sup> in the mouse) antibody (Cell Signaling Technology). Genomic DNA was extracted from paraffin blocks containing eyes or brain tumor as previously described (17). Amplification of certain transcripts from the pineal gland was accomplished by reverse transcription-PCR with the following primers: transgenic human cyclin D1, 5′-GGTCCACCTCCTCCTCCCTCTTT (forward) and 5′-GTCCTACTACC-GCCTCACACGCTT (reverse); retinal S-antigen (arrestin), 5′-GGCCGATGC-CTTCCTTTTCTCTGTT (forward) and 5′-AGCCCCATCCCCGTGACTGTGAC (reverse) used as a pineal gland–specific marker (18); Gapdh primers have been described (19). Genomic DNA was extracted from paraffin blocks containing eyes or brain tumor as previously described (17); p53 and Gapdh were amplified from DNA as described above.

Figure 1. Pineal gland hyperplasia develops in Irbp-cyclin D1 transgenic mice. A, representative photomicrographs of the pineal gland (arrow) from P10 WT and transgenic mice following immunostaining for human cyclin D1. Original magnification, ×40 and ×400 (inset). B, representative photographs of the pineal gland (arrow) on the dorsal surface of brain (top) and quantitative analysis of pineal gland size (expressed relative to WT mice at each age; bottom) from WT and transgenic mice. C, quantitative analysis of Ki67-positive pinealocytes in WT and transgenic mice of the indicated ages. Columns, mean of measurements from three to six separate mice; bars, SD. *, <i>P</i> < 0.05, compared with WT.
Histologic studies. Mice were sacrificed using CO2 or isoflurane followed by decapitation. The skull was immediately denuded, and the mandible was removed, and the base of the skull was removed. After several holes were bored near the pineal gland through the cranium using an 18-gauge needle, the skull/brain was fixed in 25 mL of 4% paraformaldehyde in PBS for 48 (P14) or 72 h (>P14). Following equilibration in 70% ethanol, tissue was embedded in paraffin using an automated processor. For mice older than 10 d, skulls were decalcified using TBD-2 decalifier (Thermo Electron Corp.) following fixation, before embedding. Pinealocyte morphology was assessed by light microscopy analysis of H&E-stained coronal sections. Pineal gland size was determined in comparable sections using an eyepiece reticule. Cell density was calculated by counting 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei in an area defined by using the eyepiece reticule to assure that the same nucleus was not counted twice.

For immunohistochemical and immunofluorescence studies, 4- to 8-μm sections cut from paraformaldehyde-fixed paraffin-embedded tissues were baked at 60°C for 30 min and then deparaffinized. Antigen retrieval was performed in a steamer at >95°C for 30 min in target retrieval buffer (pH 6.0; DAKO) for Ki67, high pH target retrieval buffer (DAKO) for cyclin D1, and citrate antigen retrieval buffer (pH 6.0) for synaptophysin (SNP) and heterochromatin-associated protein 1γ (HP1γ). Slides were incubated with anti-Ki67 antibody (Novocastra), anti-cyclin D1 (Santa Cruz Biotechnology), HA, HA epitope tag of transgenic cyclin D1. Immunohistochemical staining for human cyclin D1 at P49 in WT (a) and transgenic (b and c) pineal gland. Negative control was stained without the primary antibody. D. Immunofluorescence staining for H3-K9M and HP1γ with corresponding DAPI staining in WT. Irbp-cyclin D1, Irbp-cyclin D1, p53−/− pineal glands at P49. H3-K9M images are taken at ×400 magnification and HP1γ at ×1,000 magnification with a confocal microscope.

Figure 2. RB hypophosphorylation despite continued cyclin D1 expression in aging Irbp-cyclin D1 mice. A, immunoprecipitation and Western blotting for total RB and phospho-Ser773 in mouse RB from two representative pineal glands of WT (lanes 1 and 4) and transgenic (lanes 2, 3, 5, and 6) mice at P10 (lanes 1, 2, 4, and 5) and 2 mo (lanes 3 and 6). B, Western blotting reveals the indicated proteins in pineal gland lysates from WT (lanes 1 and 2) and transgenic (lanes 3 and 4) mice at P10 (lanes 1 and 3) and 2 mo (lanes 2 and 4). HA, HA epitope tag of transgenic cyclin D1.
or anti-SNP (DAKO) followed by biotinylated secondary antibody; bound antibody was detected using streptavidin conjugated to horseradish peroxidase and 3,3′-diaminobenzidine substrate (DAKO). Anti-HIP1γ and anti-histone H3 trimethylated at lysine 9 (H3-K9M; Upstate Laboratories) were detected with FITC and cyanine Cy2 secondary antibodies, respectively. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was done using the DeadEnd Cell Death Labeling kit (Roche). The total number of Ki67 and of TUNEL-positive cells was determined by counting representative 200× fields using a fluorescence microscope equipped with an eyepiece reticle. At this magnification, one reticle box equals 2.5 × 10^4 μm^2; the total area in the counted samples ranged from 4 to 412 reticule boxes (0.01–1 mm^2), owing to the differences in size of wild-type (WT) versus transgenic pineal gland and pineal PNET. The number of positive nuclei/area was divided by the total number of DAPI-positive nuclei/area (because the cellular density varied with the genotype) to normalize the number of Ki67- or TUNEL-positive nuclei to total cell number. Digital photomicrographs of stained sections were obtained by using an Olympus BX60 microscope equipped with a SPOT RT Slider camera (Diagnostic Instruments) or with a Zeiss 510 NLO multiphoton/confocal laser scanning microscope. Composite images were constructed using Photoshop CS2 software (Adobe Systems).

**Collection of clinical data.** This retrospective study was approved by the SJCRH Institutional Review Board. Imaging studies were retrieved for 32 children with bilateral RB diagnosed at or before 2 y of age at SJCRH between 1997 and 2003. Magnetic resonance images were reviewed from the 26 in whom the scan was (a) obtained within 6 mo of diagnosis and (b) judged to have adequate imaging of the pineal gland. Maximum pineal gland axial and anteroposterior dimensions were compared with published normal values (20).

**Statistical analysis.** Kaplan-Meier survival curves were estimated for 4 to 17 mice of each genotype. Pairwise comparisons were made in the *Irbp-cyclin D1* mice that were p53+/− (*n* = 15), p53−/− (*n* = 17), and p53+/− (*n* = 7) by using exact log-rank tests. The two-sample *t* test was used to compare mean human pineal gland sizes between children with bilateral RB and normal children <2 y of age (20) and to assess differences in quantitative measurements among mouse pineal gland samples.

**Results**

**Transgenic expression of cyclin D1 causes pineal gland hyperplasia.** We first verified that the human *cyclin D1* transgene was selectively expressed in the pineal gland but not in the adjacent brain (Fig. 1A; Supplementary Fig. S1A); mouse cyclin D1 was undetectable at this stage (Supplementary Fig. S1B). Although the transgenic mice seemed healthy, the pineal gland was visibly enlarged at 2 months of age (Fig. 1A and B, top). Quantitative analysis showed 5- and 6-fold enlargement over the WT pineal size at postnatal day (P) 10 and P49, respectively (Fig. 1B, bottom). At earlier points, the WT and transgenic pineal glands were similar in size.

Cyclin D1 drives cell proliferation by functionally inactivating RB and the related p107 and p130 (e.g., ref. 21). Consistent with this fact, pineal gland enlargement was preceded by increased pinealocyte density (Supplementary Fig. S1C) and increased proportion of cells expressing the proliferation marker Ki67 at P0 and P10 (Fig. 1C; Supplementary Fig. S2). Interestingly, the number of Ki67-positive cells decreased over time, and both WT and *Irbp-cyclin D1* pinealocytes had exited the cell cycle by P49 (Fig. 1C). Hence, ectopic expression of cyclin D1 transiently enhanced cell proliferation to cause pineal gland enlargement that did not seem to have untoward consequences.

**Cessation of proliferation in adult *Irbp-cyclin D1* mice correlates with RB hypophosphorylation despite continued cyclin D1 expression.** Cyclin D1 promotes cell proliferation by blocking RB function by two generally accepted mechanisms: inducing Cdk4- and Cdk6-dependent RB hyperphosphorylation to disrupt its functional “pocket” or titrating Cdk inhibitors such as p21Cip1 and p27Kip1 from Cdk2-containing complexes (reviewed in refs. 1, 22). By immunoprecipitating RB and immunoblotting for Cdk4(6)-dependent phosphorylated Ser277 and Ser278 (23, 24), we found that excess proliferation in the transgenic pineal gland at P10 correlated with enhanced cyclin D1–directed phosphorylation that was not observed at 2 months when proliferation had ceased (Fig. 2A; data not shown). Despite the reversal of RB phosphorylation, both immunoblotting (Fig. 2B) and immunohistochemical staining (Fig. 2C) showed that transgenic cyclin D1 expression was maintained and endogenous Cdk4 was only slightly decreased at 2 months. Hence, RB dephosphorylation and arrested pinealocyte proliferation in the aging transgenic mice did not only result from repression of the transgene or its catalytic subunit.

We considered whether the cell cycle arrest induced at 2 months might represent cellular senescence, a tumor-suppressive mechanism in which functionally active RB is known to play a role (reviewed in refs. 25–27). It colocalizes with heterochromatin-associated proteins in subnuclear foci (senescence-associated heterochromatin foci) to silence the expression of certain genes like those activated by E2Fs (28). Immunofluorescence staining showed that the HIP1γ and H3-K9M accumulated in foci in pinealocyte nuclei in 2-month-old *Irbp-cyclin D1* mice but not in...
P10 transgenic mice (data not shown) nor in 2-month-old WT mice (Fig. 2D). We conclude that decreased pinealocyte proliferation in 2-month-old Irbp-cyclin D1 mice correlates with loss of Cdk4(6)-dependent RB phosphorylation and with the formation of heterochromatin changes suggesting a state of cellular senescence.

**p53 suppresses tumor progression in the Irbp-cyclin D1 pineal gland.** The p53 tumor suppressor induces cellular senescence in response to a variety of stimuli, including oncogene expression (e.g., refs. 29, 30); as such, we used a genetic approach to define its importance in the senescent-like state we observed in the pineal gland. The pineal gland was only slightly larger at P10 in Irbp-cyclin D1, p53+/− mice compared with transgenic mice (Fig. 3B). We conclude that decreased pinealocyte proliferation and the senescence-like state that occurs as Irbp-cyclin D1 transgenic mice age.

Irbp-cyclin D1, p53+/− and Irbp-cyclin D1, p53−/− mice had markedly decreased survival compared with transgenic mice retaining WT p53 alleles (Fig. 4A) [Irbp-cyclin D1, p53+/− versus Irbp-cyclin D1, p53−/− (P = 0.0156); Irbp-cyclin D1, p53+/− versus Irbp-cyclin D1, p53−/− (P = 0.0049); and Irbp-cyclin D1, p53−/− versus Irbp-cyclin D1, p53+/− (P = 0.0001)]. These findings indicate that p53 is required for cell proliferation arrest and the senescence-like state that occurs as Irbp-cyclin D1 transgenic mice age.
an Arf−/− mouse processed in parallel (Fig. 4C; some data not shown). This indicated that the remaining p53 allele was lost in the progression to PNET.

**p53-dependent tumor suppression does not depend on p21Cip1 or p19Arf.** Oncogenic signals can activate the p53 pathway in an Arf-dependent manner and the Cdk inhibitor p21Cip1 is a key downstream effector of p53 (reviewed in ref. 33). Interestingly, Arf expression was not detectable by Western blotting or by using an ArflacZ/lacZ reporter mouse (34) at P10 or at 2 months (negative data not shown). In contrast, p21Cip1 was detectable at P10 in the Irbp-cyclin D1 pineal gland; relatively higher expression in the Irbp-cyclin D1, p53+/− pineal gland indicated that the induction at P10 was p53 dependent (Fig. 5A and B, compare lane 3 with lane 1). At 2 months when proliferation had ceased in WT and transgenic mice (Fig. 1C), p21Cip1 was not evident [Fig. 5A (lanes 2 and 4) and B (lane 2)]. The continued p21Cip1 expression in the pineal gland of 2-month-old Irbp-cyclin D1, p53−/− mice (Fig. 5B, lane 4) indicated that it was not sufficient to arrest pinealocyte proliferation at that stage.

Although the absence of p19Arf or p21Cip1 at 2 months in p53+/− transgenic mice suggested that neither was essential for cell cycle arrest and tumor suppression, we used a genetic strategy to formally evaluate their roles. As in transgenic mice retaining p21 (Figs. 1C and 3B, b), pinealocyte proliferation had ceased in Irbp-cyclin D1, p21−/− mice at 2 months of age (Supplementary Fig. S6A), with heterochromatin foci formation similar to that in Irbp-cyclin D1, p21+/− mice (Supplementary Fig. S6B). A small number (n = 3) of Irbp-cyclin D1, p21−/− mice have aged to ~5 months with no evidence of compromised survival. Similarly, 11 Irbp-cyclin D1, Arf−/− and 6 Irbp-cyclin D1, Arf+/− mice observed for an average of 9.2 (range, 2–18) months showed no evidence of brain tumor. Hence, neither p19Arf nor p21Cip1 was essential for tumor suppression in Irbp-cyclin D1 mice.

**p18Ink4c maintains cell cycle arrest and blocks tumor formation.** Recent work established that the Cdk4(6) inhibitor p18Ink4c prevents PNET in a mouse model of medulloblastoma driven by Ptc mutation (35). In our model, loss of Cdk4(6)-mediated RB phosphorylation at P49 correlated with progressively higher expression of p18Ink4c in the transgenic pineal gland (Fig. 5A), proceeding independently of p53 (Fig. 5B). At 2 months of age, very rare Ki67-positive cells (~0.05% of total) were present in WT, Irbp-cyclin D1, and nontransgenic Ink4c−/− pineal glands (Figs. 1C and 5C, top). In contrast, ~1.5% of cells in Irbp-cyclin D1, Ink4c−/− pineal glands expressed Ki67 (Fig. 5C, middle). This 30-fold increase in proliferating cells was important because Irbp-cyclin D1, Ink4c−/− mice developed pineal gland PNET as they aged to ~7 months (n = 3 of 3 mice examined; Fig. 5D). In contrast, six Irbp-cyclin D1, Ink4c−/− mice euthanized between 8 and 18 months of age had no evidence of pineal gland PNET on gross or microscopic examination. Despite the continued proliferation in a fraction of cells in the Irbp-cyclin D1, Ink4c−/− pineal gland at 2 months, immunostaining for H3-K9M showed that Ink4c was not essential for formation of senescence-associated heterochromatin changes (Fig. 5C, bottom). These findings show that Ink4c, a bona fide tumor suppressor in

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**Figure 5.** p18Ink4c preserves cell cycle arrest and blocks PNET progression in Irbp-cyclin D1 mice. A and B, Western blotting reveals the indicated proteins in pineal gland lysates from mice of the indicated genotypes at P10 and 2 mo. HA, HA epitope on the human cyclin D1. C, immunohistochemical staining for the proliferation marker Ki67 (top and middle) and heterochromatin-associated H3-K9M (bottom) in the pineal gland of the indicated mice at P49. D, representative photomicrographs showing Irbp-cyclin D1, Ink4c−/− pineal gland tumor in whole mount (a) and following H&E staining (b).
this model, was required for the maintenance of cell cycle arrest in Irbp-cyclin D1 pinealocytes, but it was not needed for formation of the heterochromatin foci associated with an initial cell cycle exit.

**Pineal gland hypertrophy in children with heritable RB.** We considered whether subclinical pineal enlargement also occurred in children with bilateral RB, who have heritable RB deficiency, and are prone to developing PNET in the pineal gland (36, 37). Review of magnetic resonance images of the brain in 26 such children showed that the pineal gland size exceeded normal in one dimension in 19 (73%) and in two dimensions in 10 (38%; two-tailed tests: \( P < 0.001 \) and \( P = 0.004 \), respectively; Fig. 6A). Only one of these patients developed frank pineoblastoma. Tissue was not available for sequencing of the p53 gene in the tumor tissue, but immunohistochemical staining indicated that p53 was detectable (Fig. 6B) and, therefore, likely mutated (38, 39). Coupled with our mouse model, these findings suggested that haploinsufficiency for RB may result in a relative excess of cyclin D1/Cdk4 activity that fosters pineal gland enlargement but only rarely progresses to PNET.

**Discussion**

Our findings show that primary disruption of the RB pathway by deregulated cyclin D1 promoted a premalignant lesion in the mouse pineal gland. p53 prevented the progression to PNET by a strikingly robust process because transgenic mouse survival was not compromised without additional germ-line genetic aberrations. This differs from many mouse models where loss of p53 accelerates tumorigenesis; here, p53 loss was required for tumor development. In our model, p53 promoted the development of a state resembling cellular senescence; however, a well-accepted regulator and effector of p53, p19Arf and p21Cip1, were both dispensable. This highlights the tissue-specific and context-dependent importance of other components of the p53 pathway. We also found that a potent inhibitor of Cdk4(6), p18Ink4c, maintained cell cycle arrest and blocked progression to PNET. Ink4c has been increasingly implicated as a tumor suppressor in experimental brain tumor models and human tumor samples (35, 40–43). Although intuitively likely, its role in blocking cyclin D1–driven neoplasia has not been previously established. Lastly, we show that subclinical pineal gland enlargement was frequent in children with heritable RB (who are at high risk for developing pineal gland PNET), essentially mimicking the new model we have developed.

That p53 played a critical role to block PNET progression in the pineal gland was surprising because it is seemingly dispensable as a tumor suppressor in the retina of these mice (14). In the pineal gland, the largest measurable effect of p53 was to induce cell cycle arrest correlating with reversal of cyclin D1–mediated RB phosphorylation and the appearance of a senescence-like state with changes in heterochromatin-bound proteins. Interestingly, this only occurred after a prolonged period of enhanced cell proliferation. Hence, the arrest was not simply a direct response to an oncogenic insult, a finding that is consistent with our observation that p19Arf was not required. Although somewhat unexpected given the current model for Arf induction as an “oncogene sensor” (44), it is not without precedent: Arf is not necessary for p53-dependent tumor suppression when SV40 T121 is expressed in the mouse choroid plexus (45). We can propose two alternative explanations for how p53 is engaged. First, cyclin D1 might activate p53 in an oxidative stress–dependent or DNA damage–dependent manner, bypassing Arf altogether (reviewed in ref. 46). Alternatively, p53-dependent arrest might represent a developmental process intersecting with and blocking the oncogenic signals. Such an explanation would be consistent with the observation that genetically susceptible children (like those with...
heritable RB) are at highest risk of developing pineal gland PNET in the first several years of life (36, 37).

Several recent articles have begun to address the role of Ink4c as a tumor suppressor in brain neoplasms. Zindy and colleagues (41) showed that loss of one or both Ink4c alleles is needed to foster cerebellar PNET formation in p53-/- mice; hence, a collaborative interaction exists. Members of the Roussel laboratory also established that Ink4c loss enhances cerebellar PNET driven by Shh pathway activation (due to Ptc mutation; ref. 35). However, cyclin D1 is only one of several oncogenes when Shh is deregulated (35, 47). Better definition of the relationship between cyclin D1 and p18Ink4c stems from the finding that ectopic cyclin D1 expression can drive tumor formation only in Ink4c-null mouse granule neuron precursors following orthotopic implantation (42). Our in vivo findings in a transgenic model complement this growing body of evidence by establishing that inactivation of either Ink4c or p53 was necessary and sufficient for PNET progression when cyclin D1 represented the principal oncogenic signal. Parenthetically, tumor suppression by Ink4c did not apply to the retina as the Irbp-cyclin D1 ocular phenotype was unaffected by Ink4c (or p21) loss (Supplementary Fig. S7); hence, cellular context must be considered.

Our findings highlight the complex relationships between the RB and p53 pathways. In the absence of p53, p18Ink4c expression increased but still was not sufficient to prevent PNET. Although we have not evaluated p53 function in the Irbp-cyclin D1, Ink4c-/- tumors, it remains intact in cerebellar PNETs occurring in Ptc+/C0, Ink4c-/- mice (35). Although both genes are needed for tumor suppression in our model, the basis for the interdependence is not clear from our studies. The two proteins did not seem to lie in a simple linear pathway because a greater fraction of pinealocytes proliferated at 2 months in the absence of p53 than in the absence of Ink4c (42% versus 1.5%, respectively). In addition, the latency for tumor development was longer in Irbp-cyclin D1, Ink4c-/- mice (~7 months) than Irbp-cyclin D1, p53-/- mice (3-4 months). Finally, one measure of p53 activity, p21Cip1 induction, was dampened at 2 months after pinealocytes arrest, whereas p18Ink4c expression was high at P10 and even higher when the cells had arrested. The simplest explanation is that p53 and p18Ink4c represent parallel pathways that check pinealocyte proliferation by fundamentally different mechanisms. We can also speculate that the two genes might act sequentially such that p53 drives the cyclin D1-expressing pinealocytes into an arrested state, and p18Ink4c maintains it. However, this concept needs to be evaluated experimentally.

Approximately 10% of children with heritable RB develop a metachronous brain PNET, commonly pineoblastoma (36, 37). That this only occurs in a subset and usually several years after the diagnosis of RB implies that RB inactivation alone is insufficient for tumorigenesis in PNETs. Although our mouse model of deregulated cyclin D1 expression may not totally equate to RB haploinsufficiency in children, we found parallel pineal gland enlargement in children with heritable RB. In these children, progression from pineal gland enlargement to PNET might be halted in a p53- or Ink4c-dependent manner. Due to the aggressive nature of this tumor (currently the leading cause of death in these children in the first decade of life (36, 37)), preventive therapy for children known to be at high risk is an attractive goal. Our finding of Ink4c-mediated tumor suppression in pineal PNET when RB function is compromised suggests that pharmacologic blockade of Cdk4(6) activity may be a useful strategy to block progression of premalignant lesions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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