Cooperation between Cdk4 and p27^{kip1} in Tumor Development: A Preclinical Model to Evaluate Cell Cycle Inhibitors with Therapeutic Activity

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Abstract

Deregulation of the G_1-S transition of the cell cycle is a common feature of human cancer. Tumor-associated alterations in this process frequently affect cyclin-dependent kinases (Cdk), their regulators (cyclins, INK4 inhibitors, or p27^{Kip1}), and their substrates (retinoblastoma protein). Although these proteins are generally thought to act in a linear pathway, mutations in different components frequently cooperate in tumor development. Using gene-targeted mouse models, we report in this article that Cdk4 resistance to INK4 inhibitors, due to the Cdk4 R24C mutation, strongly cooperates with p27^{Kip1} deficiency in tumor development. No such cooperation is observed between Cdk4 R24C and p18^{INK4c} absence, suggesting that the only function of p18^{INK4c} is inhibiting Cdk4 in this model. *Cdk4*$^{R/R}$ knock in mice, which express the Cdk4 R24C mutant protein, develop pituitary tumors with complete penetrance and short latency in a *p27*$^{Kip1-/-}$ or *p27*$^{Kip1+/-}$ background. We have investigated whether this tumor model could be useful to assess the therapeutic activity of cell cycle inhibitors. We show here that exposure to flavopiridol, a wide-spectrum Cdk inhibitor, significantly delays tumor progression and leads to tumor-free survival in a significant percentage of treated mice. These data suggest that genetically engineered tumor models involving key cell cycle regulators are a valuable tool to evaluate drugs with potential therapeutic benefit in human cancer. (Cancer Res 2005; 65(9): 3846-52)

Introduction

Cell cycle progression relies on the activation of cyclin-dependent kinases (Cdk), which successively act in G_1 to initiate S phase and in G_2 to initiate mitosis. Upon mitogenic stimuli, D-type cyclins are induced and bind to and activate the cell cycle kinases Cdk4 and Cdk6 (reviewed in ref. 1). Cyclin D-Cdk4/6 complexes phosphorylate and partially inactivate pRb, allowing the expression of some E2F-target genes such as the gene encoding cyclin E. Induction of cyclin E, in turn, allows the activation of Cdk2, which is also able to further phosphorylate and completely inactivate pRb, releasing a massive transcription process that results in the expression of genes required for DNA replication and mitosis. Cdk2 is also able to bind A-type cyclins during S phase, whereas control of G_2 and M phases is mainly dependent on cyclin A-Cdk1 and cyclin B-Cdk1 complexes. Whether these Cdks have essential roles or could be compensated by other kinases has been challenged by recent work using mice deficient in Cdk4 and Cdk6 or Cdk2 (2–4).

To prevent abnormal proliferation, cyclin-Cdk complexes are regulated by two families of cell cycle inhibitors, the INK4 and Cip/Kip proteins, that block Cdk kinase activity (reviewed in refs. 5, 6). INK4 proteins (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}) specifically bind to Cdk4 or Cdk6 proteins disturbing their interaction with D-type cyclins leading to a kinase-inactive state. Cip/Kip inhibitors (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}), on the other hand, bind cyclin-Cdk complexes forming ternary structures that also lack kinase activity (reviewed in ref. 7). Although Cip/Kip proteins are able to bind all cell cycle Cdks, they seem to preferentially target Cdk2 (6, 8). Among these proteins, p27^{Kip1} (9–11) displays a unique pattern of responsiveness to a wide variety of mitogenic and antimitogenic signals, which makes it quite distinct from the other Cip/Kip members. p27^{Kip1} mediates the growth arrest induced by transforming growth factor β, contact inhibition, growth in suspension, cyclic AMP agonists, and other signals (8).

The involvement of these cell cycle inhibitors in cancer has been extensively established in the last years (1, 12, 13). Loss of p16^{INK4a}, p15^{INK4b}, and p18^{INK4c} activity by mutation, deletion, and/or promoter methylation has been observed in a variety of human tumors and the respective deficient mice display an increased susceptibility to tumor development. Whereas p16^{INK4a}- and p15^{INK4b}-deficient mice only develop few spontaneous tumors with long latency (14–16), p18^{INK4c}-deficient mice develop pituitary tumors with high penetrance (14, 17). The Cdk4 R24C mutation, found in human melanoma, renders a Cdk4 protein insensitive to the INK4 inhibitors (18). The presence of the Cdk4 R24C mutant protein could therefore be equivalent to the inactivation of multiple INK4 inhibitors. Knock in mice carrying this mutation develop a wide spectrum of tumors with complete penetrance (19, 20).

Alteration of the *Cip/Kip* genes is a rare event in human primary tumors and cancer cell lines. Yet, p27^{Kip1} protein levels are frequently decreased in human malignancies due to abnormal proteolytic degradation (8), and p27^{Kip1} inactivation is an independent prognostic marker in many human cancers correlating with higher tumor grade and poor survival (reviewed in refs. 21, 22). p27^{Kip1}-deficient mice are significantly bigger in weight than wild-type mice, exhibit organ hypertrophy, and also develop pituitary tumors later in life (23–25). In addition, p27^{Kip1} deficiency is able to cooperate with other genetic alterations, such as PTEN deficiency, or chemical carcinogens in tumor development in the mouse (26–28).

We have analyzed the effect of inactivating both INK4 and Kip pathways in tumor suppression. To this end, we have crossed Cdk4

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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R24C knock in mice with $p27^{Kip1}$- or $p18^{INK4c}$-deficient animals. The Cdk4 R24C mutant does not cooperate with $p18^{INK4c}$ deficiency in agreement with a specific role of $p18^{INK4c}$ in Cdk4 inhibition. In contrast, Cdk4 activation strongly cooperates with $p27^{Kip1}$ deficiency in tumor development in the pituitary and other tissues. Because these tumors are initiated by inactivation of the INK4 and Cip/Kip inhibitory pathways (19, 23–25), we have analyzed the effect of a small-molecule Cdk inhibitor, flavopiridol, on the growth of these pituitary tumors. Whereas nontreated double mutant mice ($Cdk4^{R/R}$;$p27^{+/-}$) mice die of pituitary and other tumors at 6 to 7 months, continuous treatment with flavopiridol for 3 weeks results in a significant delay in tumor development and the absence of pituitary tumors in 20% of treated mice after 1 year.

Materials and Methods

Mice and pathologic analysis. $Cdk4^{R/R}$ mice expressing an endogenous Cdk4 R24C protein, and $p18^{INK4c}$-*null* and $p27^{Kip1}$-deficient mice were reported previously and they were genotyped as described (14, 25, 29). Mice were housed in a pathogen-free barrier area and were sacrificed in accordance with the Guidelines for Humane Endpoints for Animal Used in Biomedical Research. All animal experiments were done under the experimental protocol approved by Centro Nacional de Investigaciones Oncológicas' (CNIO's) Institutional Committee for Care and Use of Animals. Mice were maintained in a mixed 129/Sv × CD1 × C57BL6/J background at the CNIO following the animal care standards of the institution that complies with European legislation on the care and use of animals, NIH guidelines for the use of laboratory animals, and related codes of practice. All animals were observed on a daily basis. They were sacrificed at any sign of disease and normal or pathologic tissue samples were recovered for histologic and molecular analysis.

Formalin-fixed pituitary samples were stained with antibodies against adrenocorticorticotropic hormone, follicle-stimulating hormone (FSH), growth hormone, prolactin, luteinizing hormone, and thyroid-stimulation hormone (TSH, kindly provided by A.F. Parlow, National Hormone and Pituitary Program, Harbor, University of California at Los Angeles Medical Center), $p27^{Kip1}$ (Neomarkers, Fremont, CA) and the Ki67 antigen (Dako, Carpinteria, CA). Detection of apoptotic cells on tissue sections was carried out using the terminal deoxynucleotidyl transferase–mediated nick-end labeling assay (apoptag peroxidase, Intergen, Burlington, MA). Endocrine tumors of the pancreas were stained with antibodies against insulin, glucagon, somatostatin (Dako, Carpinteria, CA) and pancreatic polypeptide (Monosan, Uden, the Netherlands).

Flavopiridol treatment and nuclear magnetic resonance analysis. Flavopiridol [(−)-*cis*-2-(2-chlorophenyl)-5,7-dihydroxy-8-[4-(3-hydroxy-1-methyl) piperidinyl]-4*H*-1-benzopyran-4-one hydrochloride; NSC 649890] was supplied by Eli Lilly (Madrid, Spain) and the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Bethesda, MD). A flavopiridol stock solution of 100 mg/mL was prepared in DMSO. For daily injections of laboratory mice, a working solution of 1 mg/mL was freshly prepared with sterile PBS. Mice were randomly separated in the control and test groups, which were injected i.p. with PBS (1% DMSO) or flavopiridol (5 mg/kg per day), respectively. Test mice were subjected to a single injection during five consecutive days in 1 week (1-week treatment) or 3 weeks in a row (3-week treatment; five injections per week). Statistical significance was assessed using the log-rank test of Kaplan-Meier analysis (SPSS, Inc., Chicago, IL).

For nuclear magnetic resonance (NMR) analysis, all animals were i.p. anesthetized with a mixture of ketamin (75 mg/kg) and medetomidine (1 mg/kg). After resonance analysis for no more than 30 minutes, all animals were s.c. inoculated with atipamezol (2 mg/kg) to reverse the effect of the anesthesia. All NMR experiments were done on a 4.7 T/40 cm horizontal bore magnet interfaced to a Bruker Paravision console (Bruker, Ettlingen, Germany), and with standard gradient coil capable of a 200 μs rise time and 300 mT/m maximum gradient strength. For acquisition, an in-house-made six-turn solenoidal coil (2.5 diameter × 2.5 cm length) was used for both RF transmission and reception. The coil was specifically mounted on a cylinder inside a customized semicylindrical plexiglass assembly, which allows one to reproducibly and quickly placing the mouse head in prone position inside that coil holder and calculated shape and dimensions to situate the animal cradle into the gradient coil and magnet isocenter. Single sagital, coronal, and transversal images were obtained by a fast gradient echo sequence to localize the subsequent three-dimensional T_2-weighed coronal images, as measured by a standard turbo spin echo. The sequence variables were TE_{eff} = 42 ms, TR = 2,500 ms, matrix = 64 × 256 × 16, FOV = 3 × 3 × 1.2 cm, turbo factor = 8, and number of acquisitions = 2. The three-dimensional reconstruction of the tumor structure was done based on these sets of images (ImageJ; NIH, http://rsb.info.nih.gov/ij). Contour lines of the complete regions of interest were traced by selection of threshold, and roto-translated using internal fiduciary markers. These contour lines were overlapped, stacked, and stereo-cine rendered with the VolumeJ plugin (M. Abràmoff; http://www.isi.uu.nl/people/michael/vr.htm). Three-dimensional images were composed and pseudo-colored using Quicktime Pro (rel. 6.5) for Macintosh (Apple Computer, Inc., Cupertino, CA).

Results

Combination of Cdk4-, $p18^{INK4c}$-, and $p27^{Kip1}$-targeted alleles. The presence of the Cdk4 R24C mutant does not compromise embryonic development in a $p27^{Kip1}$-deficient ($p27^{-/-}$) or $p18^{INK4c}$-deficient ($p18^{-/-}$) background. $Cdk4^{R/R}$;$p27^{-/-}$ or $Cdk4^{R/R}$;$p18^{-/-}$ mice are born at the expected Mendelian ratio and do not display major abnormalities at birth. However, whereas single $Cdk4^{R/R}$ or $p27^{-/-}$ mutants are significantly bigger than wild-type littermates (about 20% bigger in weight at 6 weeks of age), combination of these two genotypes results in dramatically smaller animals (Fig. 1). Double $Cdk4^{R/R}$;$p27^{-/-}$ mutants are shorter (Fig. 1*B*) and smaller in weight than wild-type mice (about 75% at 6 weeks of age) or single mutants (about 60%; Fig. 1*C*). This phenotype is specific for the double mutants because $Cdk4^{R/R}$;$p27^{+/-}$ mice are similar to single mutants. $Cdk4^{R/R}$;$p27^{-/-}$ double mutants also display other pathologic deficiencies such as curved spinal cord and lordokyphosis (hunchbacked spine; Fig. 1*D*). The reason for these phenotypes is not clear at this moment although the smaller size is likely to be related to the disturbed morphology of the pituitary gland in those animals (see below).

No significant differences in size or weight are found between $Cdk4^{R/R}$;$p18^{-/-}$ double mutants and the single mutants (data not shown). In addition, no cooperation is observed between these two mutations because all single ($Cdk4^{R/R}$;$p18^{+/+}$ and $Cdk4^{+/+}$;$p18^{-/-}$) or double ($Cdk4^{R/R}$;$p18^{-/-}$) mutants have a similar survival curve (Fig. 2*A*) and most of animals die in the second year of life with similar pathologies and tumor spectrum to those previously described for the single mutants (14, 19; data not shown).

Similar to the Cdk4 R24C-expressing or the $p18^{INK4c}$-deficient mice, $p27^{-/-}$ single mutants have a decreased survival due to the presence of pituitary adenomas and most of them are dead by 80 weeks. $Cdk4^{R/R}$;$p27^{-/-}$ double mutants, on the other hand, are viable in the first weeks of life but their survival drops dramatically at 8 to 10 weeks of age (Fig. 2*B*). No mouse with this genotype survives longer than 11 weeks. The intermediate genotypes carrying one normal allele of *Cdk4* ($Cdk4^{+/R}$;$p27^{-/-}$) or $p27^{Kip1}$ ($Cdk4^{R/R}$;$p27^{+/-}$) display intermediate behaviors, whereas double heterozygous mice ($Cdk4^{+/R}$;$p27^{+/-}$) are indistinguishable from the single mutants (Fig. 2*B*).

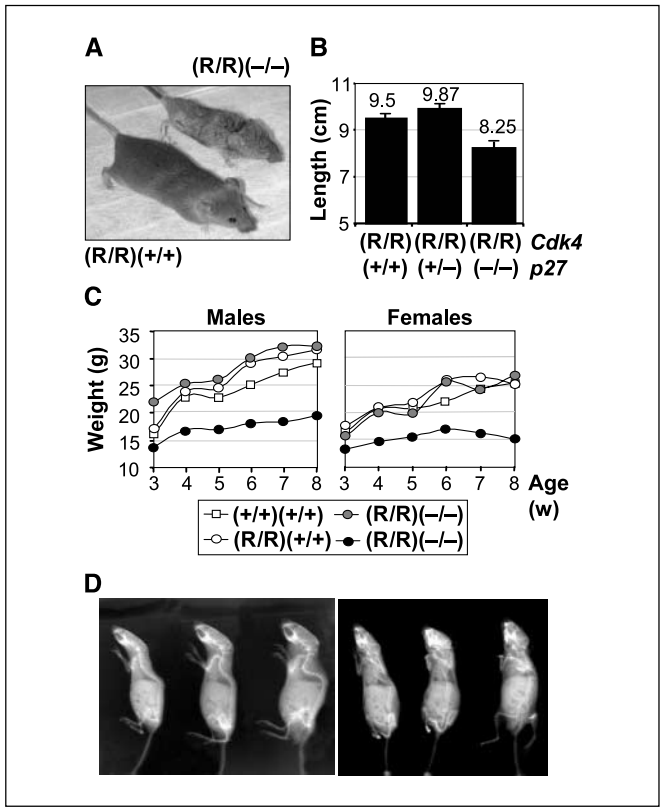

Figure 1. Morphologic abnormalities in $Cdk4^{R/R};p27^{-/-}$ mice. *A,* $Cdk4^{R/R};p27^{-/-}$ double mutant [(R/R)(−/−)] versus a $Cdk4^{R/R};p27^{+/+}$ mouse [(R/R)(+/+)]. *B,* length of different genotypes from the tip of the nose to the proximal end of the tail at 8 weeks of age. *C,* weight of males and females of different genotypes at 3 to 8 weeks of age. *D,* pathologic curvature of the spinal cord in three $Cdk4^{R/R};p27^{-/-}$ mice in a lateral (*left*) or coronal (*right*) view.

Cooperation between Cdk4, $p18^{INK4c}$, and $p27^{Kip1}$ in tumor development. Pathologic examination of these animals revealed a strong cooperation between the Cdk4 mutant and $p27^{Kip1}$ deficiency in tumor development. All $Cdk4^{R/R};p27^{-/-}$ animals developed pituitary tumors, and other malignancies were also observed with diverse incidence in these animals (Supplementary Table S1). Double $Cdk4^{R/R};p27^{-/-}$ mutants developed hyperplasia of the adrenal gland and thyroid C cells, pheochromocytoma and tumors of pancreatic β cells and Leydig cells, and adenocarcinoma of the lung. All these pathologies have been described in the single $Cdk4^{R/R}$ or $p27^{-/-}$ mutants although with significantly higher latency (19, 23–25, 30). In addition, these double mutants as well as the double heterozygous $Cdk4^{+/R};p27^{+/-}$ mice also developed other pathologies such as thyroid follicular adenoma and lymphomas (Supplementary Table S1 and Fig. 3). Only a low incidence of lymphomas (<5%) is observed in age-matched wild-type mice with similar genetic background.

Pituitary tumors in $Cdk4^{R/R}$ mice develop in the pars distalis (adenohypophysis; Fig. 4*C-D*) and only rarely affect pars intermedia (19). $p27^{Kip1}$-deficient mice, however, develop pars intermedia tumors of the pituitary with similar origin to those in $pRb^{+/-}$ and $p18^{-/-}$ single mutant mice (14, 17, 31, 32). Lost of one $p27^{Kip1}$ allele in a $Cdk4^{R/R}$ background provokes the simultaneous development of pars intermedia and pars distalis adenomas (Fig. 4*E-F*). Tumors in $Cdk4^{R/R};p27^{-/-}$ double mutants are more difficult to classify because they are frequently heterogeneous, hemorrhagic, and poorly differentiated. In most of cases, these tumors are composed of hyperplasia or well-differentiated adenoma of the pars intermedia cells along with a highly vascular neoplasm most probably derived from the pars distalis. These undifferentiated regions are formed of small sized endocrine-like cells with scant cytoplasms surrounding ectasic vascular channels or forming trabecula (Fig. 4*G-H*). Frequently, these regions contain scarce large cells of unknown lineage (Fig. 4*I-J*). In addition, we have detected TSH-, prolactin-, and FSH-expressing cells in some of the undifferentiated tumors in agreement with an adenohypophysis origin of these malignancies (Fig. 4*K-L*).

Treatment of $Cdk4^{R/R};p27^{-/-}$ and $Cdk4^{R/R};p27^{+/-}$ pituitary tumors with flavopiridol. Because $Cdk4^{R/R};p27^{-/-}$ tumors develop with complete penetrance and short latency, we used these animals for preclinical studies with flavopiridol, a small-molecule Cdk inhibitor used as a model for therapeutic inhibition of the cell cycle (reviewed in ref. 33). Flavopiridol was given to 4-week-old mice by 1 week as indicated in Materials and Methods. Mice subjected to this treatment survive longer (an average of 10 weeks [n = 8] versus 8 in nontreated mice [n = 25]; P = 0.006) although all of them died by 12 weeks of age (Fig. 5*A*).

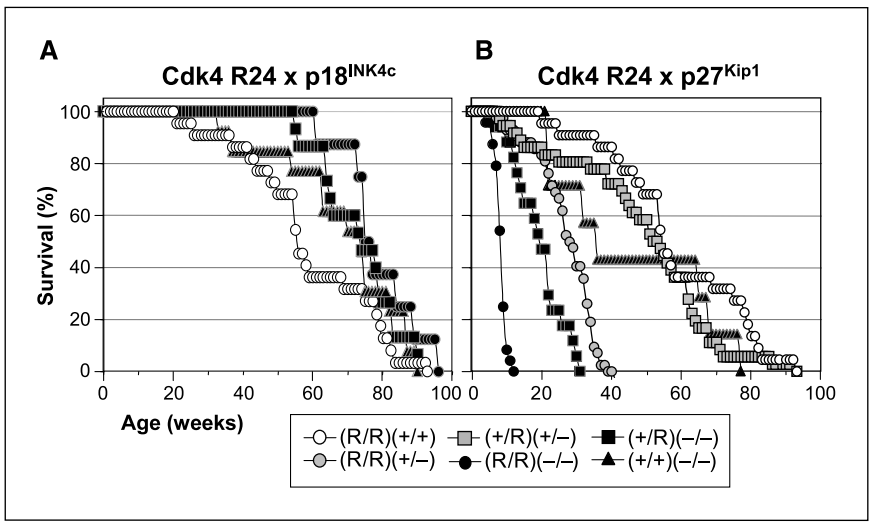

Figure 2. Survival curve of mice carrying targeted alleles of *Cdk4*, $p18^{INK4c}$, or $p27^{Kip1}$. *A,* survival of $Cdk4^{R/R};p18^{+/+}$ (○, n = 22), $Cdk4^{+/+};p18^{-/-}$ (▲, n = 13), $Cdk4^{+/R};p18^{-/-}$ (■, n = 15), and $Cdk4^{R/R};p18^{-/-}$ (●, n = 8) mice. *B,* survival curve of $Cdk4^{R/R};p27^{+/+}$ (○, n = 22), $Cdk4^{R/R};p27^{+/-}$ (◎, n = 42), $Cdk4^{R/R};p27^{-/-}$ (●, n = 24), $Cdk4^{+/R};p27^{+/-}$ (□, n = 36), $Cdk4^{+/R};p27^{-/-}$ (■, n = 17), and $Cdk4^{+/+};p27^{-/-}$ (▲, n = 7).

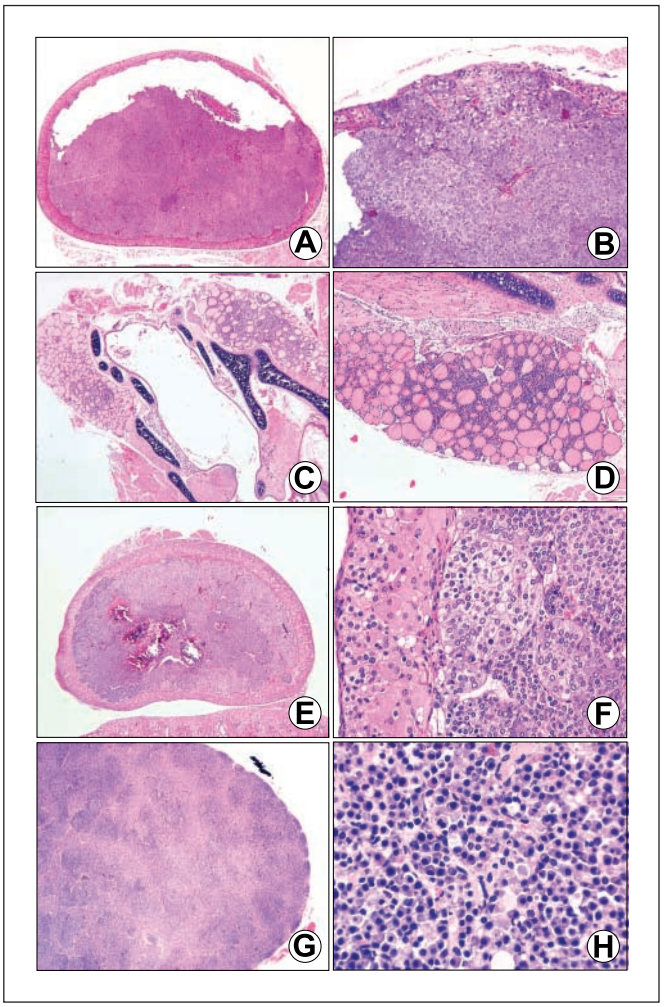

Figure 3. Representative photomicrographs of tumors developed in Cdk4$^{R/R}$; p27$^{+/-}$, Cdk4$^{+/R}$;p27$^{-/-}$, and Cdk4$^{+/R}$;p27$^{+/-}$ mice as in Supplementary Table 1. *A* and *B,* pheochromocytoma; the cortex is thinner and focally destroyed by medullar cells spreading to the capsula. *C* and *D,* thyroid C cell hyperplasia. *E* and *F,* medullar hyperplasia. *G,* B-cell lymphocytic lymphoma in the spleen. *H,* plasma cell lymphoma in a lymph node. H&E staining. Original magnification: 20× (*A*, *E*, and *G*), 40× (*C*), 100× (*D*), 200× (*B*), 400× (*F* and *H*).

A second cohort of double *Cdk4*$^{R/R}$;*p27*$^{-/-}$ mutants was exposed to flavopiridol for 3 weeks (five injections a week). As shown in Fig. 5*A*, this treatment further improves viability of these mutants. All treated animals were alive by 10 weeks of age, whereas >80% of nontreated mice were dead at this age. Average survival is 11.3 weeks in treated mice (n = 13) versus 8 weeks in nontreated mice (n = 25), representing a more statistically significant delay in the onset of death (P < 0.001).

The transient effect observed after flavopiridol treatment was due to decreased proliferation and a slight increase in apoptosis. Thus, a 4-fold reduction in proliferation rate, as measured by Ki67 staining, was detected in pituitary cells 2 to 3 weeks after the flavopiridol treatment (Fig. 5*B*). Similarly, some scarce apoptotic cells (1-2 per microscopic field at 400×) were detected in treated but not untreated tumors (Fig. 5*B*). This antiproliferative effect was transient because most treated and nontreated mice died of pituitary tumors with similar tumor sizes. Indeed, the increased rate of proliferation of pituitary cells was restored at 12 to 14 weeks, >6 weeks after the flavopiridol treatment (data not shown).

Because pituitary tumors in *Cdk4*$^{R/R}$;*p27*$^{-/-}$ mice develop rapidly and the therapeutic window is reduced, we used *Cdk4*$^{R/R}$;*p27*$^{+/-}$ mice. These animals have a half-life of about 28 weeks and none of them survive for >40 weeks of age (Fig. 2*B*). Fifteen *Cdk4*$^{R/R}$;*p27*$^{+/-}$

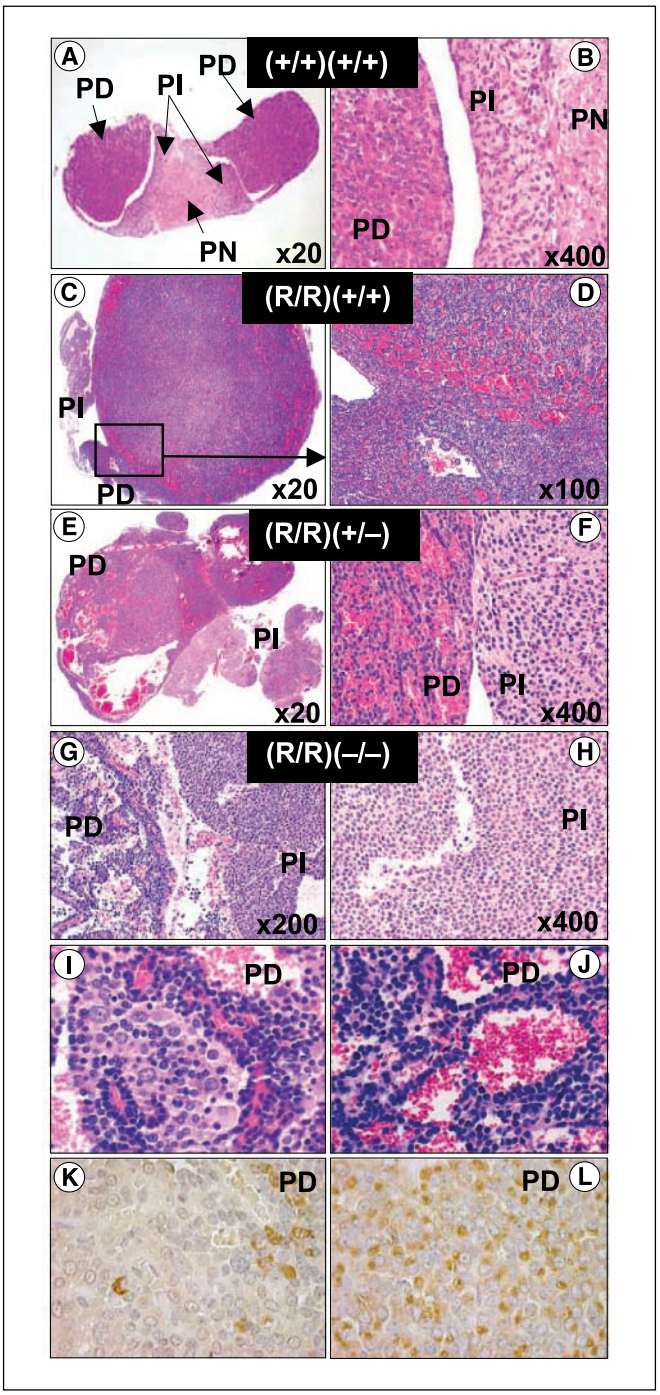

Figure 4. Representative abnormalities in the pituitary gland. *A-B,* morphology of a normal pituitary gland. *C-D,* pars distalis tumors in Cdk4$^{R/R}$;p27$^{+/+}$ mice. *E-F,* double pars distalis and pars intermedia tumors in Cdk4$^{R/R}$;p27$^{+/-}$ mice. Both proliferative components do not mix each other. *G-J,* mixed tumors in Cdk4$^{R/R}$;p27$^{-/-}$ mice composed of undifferentiated cells from the pars distalis and pars intermedia adenomas. *K* and *L,* immunohistochemical detection of TSH-positive (*K*) and FSH-positive (*L*) cells in Cdk4$^{R/R}$;p27$^{-/-}$ undifferentiated tumors. Pars distalis (*PD*), pars intermedia (*PI*), pars nervous (*PN*). Magnification: 20× (*A*, *C*, and *E*), 100× (*D*), 200× (*G*), 400× (*B*, *F*, and *H*), and 630× (*I-L*).

mice were subjected to the 3-week treatment, whereas 31 mice remained untreated. First injection was given at 24 weeks of age (Fig. 6*A*), a time when pituitary tumors are already developing in *Cdk4*$^{R/R}$;*p27*$^{+/-}$ mice.[6] In fact, part of the *Cdk4*$^{R/R}$;*p27*$^{+/-}$ colony (about 16% of the animals) is already dead of pituitary tumors at this stage (Fig. 2*B*). Survival of these mice is indeed severely compromised because all of nontreated animals die in the next 13 weeks (Fig. 6*A*). However, 1 week after the last injection of flavopiridol, mortality is clearly delayed in treated mice during the following 10 weeks. Average survival increased from 31 weeks in nontreated mice (n = 31) to 39 weeks in treated mice (n = 15), representing a statistically significant difference between both groups of animals (P < 0.001). Only one animal died from weeks 28 to 38 after flavopiridol treatment, whereas all nontreated mice died in the same period (Fig. 6*A*).

To understand the evolution of pituitary growth during the therapeutic treatment, we followed pituitary size using magnetic resonance imaging. Half of the treated and nontreated mice were analyzed every 2 weeks starting at 20 weeks of age. As shown in Fig. 6*B*, nontreated pituitaries grew rapidly up to about 22 mm^3, when most animals died. However, growth of treated pituitaries is significantly delayed by flavopiridol from week 20 to week 40. Moreover, 3 of the 15 treated mice did not show any sign of disease and were alive after >1 year of life. These three animals were sacrificed at 60 weeks of age for a complete pathologic analysis. Pituitary gland in these animals displayed a size similar to wild-type pituitaries of the same age. In fact, histologic examination of these pituitaries showed a normal organ structure and cellular morphology (Fig. 6*D-I*). No other tumor tissue was observed in these animals after careful examination of the major organs (data not shown).

Discussion

Although the simplistic scheme of the pRb pathway places all the G_1-S Cdks and their regulators upstream of pRb, there is genetic evidence suggesting that the situation is not as simple. p27^{Kip1} depletion cooperates with pRb inactivation or p18^{INK4c} absence in tumor development (17, 34). Lack of cooperation between Cdk4 and p18^{INK4c} (Fig. 2*A*) indicates that the only function of p18^{INK4c} is inhibiting Cdk4, at least in pituitary tumor suppression. Double *Cdk4*$^{R/R}$;*p18*$^{-/-}$ mutant mice seem to display an increased viability at least between weeks 50 and 70 when compared with *Cdk4*$^{R/R}$;*p18*$^{+/+}$ mice (Fig. 2*A*). Although these results could suggest that p18^{INK4c} absence might be protecting mice from the Cdk4 R24C mutation, the reduced number of *Cdk4*$^{R/R}$;*p18*$^{-/-}$ mice (n = 8) limit the relevance of these data. The fact that Cdk4 does not cooperate with p18^{INK4c} deficiency but strongly synergizes with p27^{Kip1} absence (Fig. 2) clearly delimitates two overlapping pathways for tumor suppression: the INK4-Cdk4-pRb pathway and another molecular route where p27^{Kip1} is a central regulator. The nature of the other components of the p27^{Kip1} pathway is not clear yet. Possible partners for the p27^{Kip1} pathway are cyclin E and Cdk2. However, the involvement of these molecules in the very same pathway has not been shown genetically. Cdk2 activity is not clearly up-regulated in p27^{Kip1} knock out mice (23–25 and our unpublished results) and cyclin E up-regulation synergizes with p27^{Kip1} deficiency in tumor progression suggesting that these two alterations are not functionally identical (35). In addition, Cdk2 is not essential for mitotic cell cycle (2, 3), whereas the lack of E-type cyclins suppress the M-G_1 transition (36, 37).

The function of p27^{Kip1} as a universal Cdk inhibitor was challenged by recent evidence indicating that the Cip/Kip proteins could participate in Cdk4 and Cdk6 protein stability (6). These data suggested a dual role for Cdk4 in pRb phosphorylation and a new, kinase-independent, function of Cdk4 as a Cdk2 activator through p27^{Kip1} sequestering. In addition, the fact that many tumors display reduced levels of p27^{Kip1} rather than complete inactivation may suggest that low levels of p27^{Kip1} may be more favorable to transformed cells by allowing the assembly of complexes between D-type cyclins and Cdk4 and Cdk6, without inhibiting Cdk2 complexes (8). This hypothesis is supported by a mouse model showing that p27$^{+/-}$ mammary epithelium is more susceptible to oncogene-induced tumorigenesis than p27$^{-/-}$ mammary glands at least in cooperation with particular oncogenes (38). Apart from this particular case, p27^{Kip1} and PTEN deficiency cooperate in tumor development and p27^{Kip1}-null mice are more susceptible to

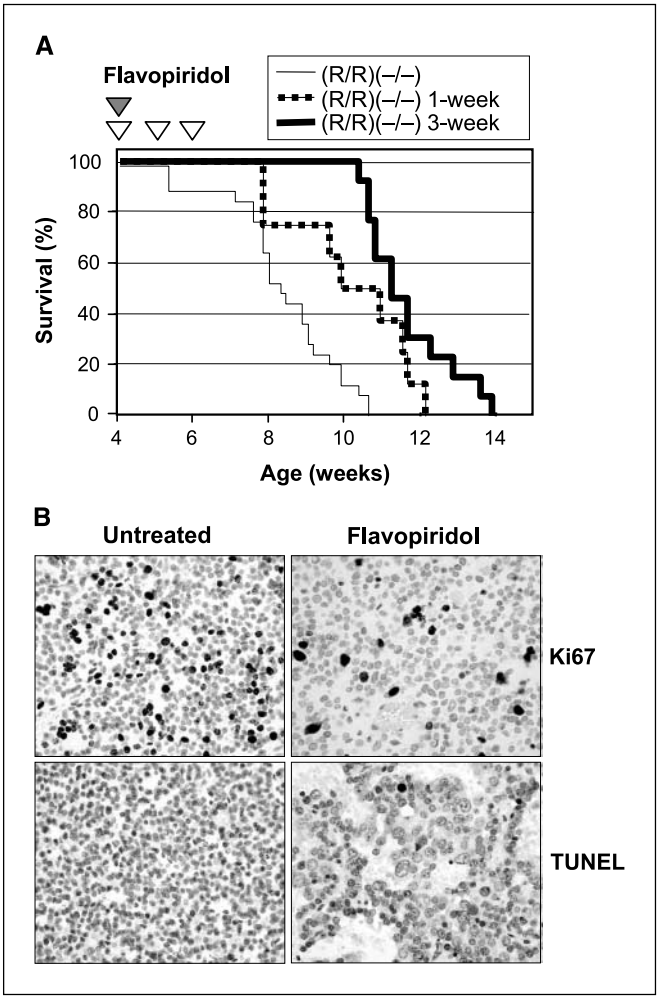

Figure 5. Tumor-free survival after flavopiridol treatment. *A,* randomly selected Cdk4$^{R/R}$;p27$^{-/-}$ mice were treated with flavopiridol for 1 week (n = 11), three continuous weeks (n = 13), or left untreated (n = 18). *B,* Ki67-positive and terminal deoxynucleotidyl transferase–mediated nick-end labeling (*TUNEL*)–positive cells in pituitary tumors untreated or analyzed 2 weeks after the treatment with flavopiridol. Magnification 400×.

[6] Unpublished observations.

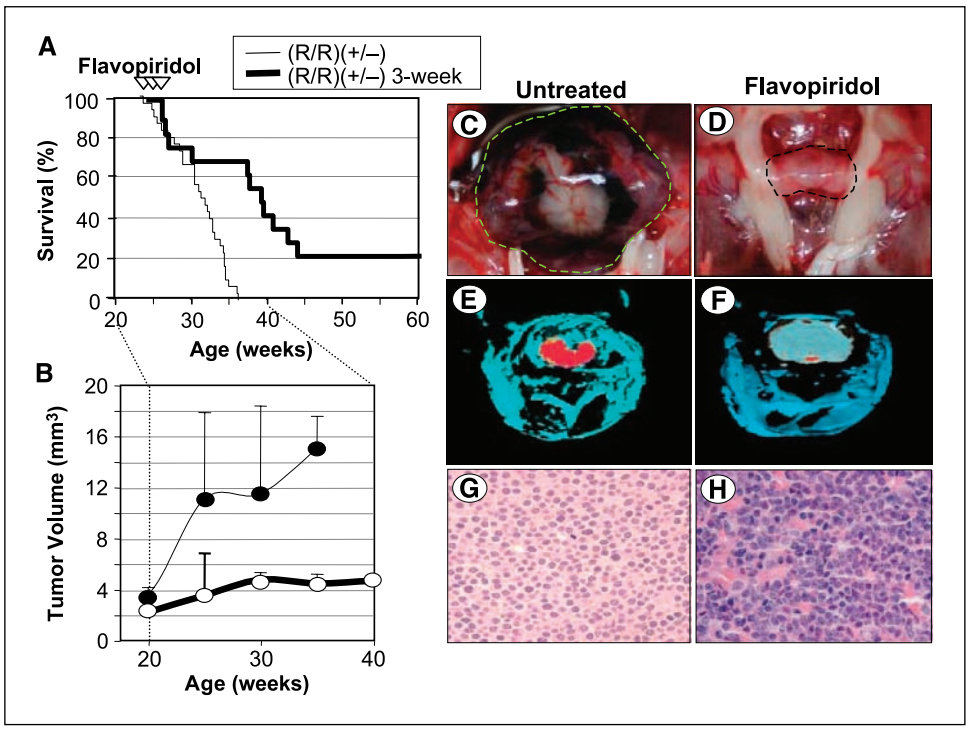

Figure 6. Effect of flavopiridol on Cdk4$^{R/R}$;p27$^{+/-}$ tumors. *A,* randomly selected Cdk4$^{R/R}$;p27$^{+/-}$ mice were treated with flavopiridol for 3 weeks (n = 15), or left untreated (n = 32). *B,* volume of treated (○) or untreated (●) tumors was monitorized by magnetic resonance imaging from the start of the treatment (when the mice were 20 weeks old) up to 40 weeks of age. *C-H,* representative images for untreated (*C*, *E*, and *G*) and treated (*D*, *F*, and *H*) mice. *C* and *D,* macroscopic pictures of the pituitary and (*E* and *F*), corresponding magnetic resonance imaging views (*red*). *G* and *H,* histology section of these pituitary glands stained with H&E (400×).

carcinogenic treatments (26–28). The fact that Cdk4 activation and p27^{Kip1} deficiency strongly cooperate in tumor development in the pituitary and other organs suggests that sequestering p27^{Kip1} is not the major role of Cdk4 in these tissues and that p27^{Kip1} is not essential for the stability of Cdk4 complexes.

Considerable evidence has accumulated in the last years suggesting that deregulation of the G_1-S transition of the cell cycle is a hallmark of human cancers (reviewed in refs. 1, 39, 40). Some tumors have alterations in the pRb locus through deletion, point mutations, or hypermethylation of its promoter. However, most other tumors have mutations that directly or indirectly deregulate Cdk activity. Some of these tumors have lost INK4 or Cip/Kip function through gene deletion, mutation, or promoter methylation (12), or have decreased protein stability (8). In some specific cases, INK4 function is lost through mutations in Cdk4 or Cdk6 that render these kinases insensitive to INK4 inhibitors (12). In all these cases, the primary effect of these mutations is the increased activation of G_1-S Cdks: Cdk4, Cdk6, and Cdk2. In fact, overexpression of Cdk inhibitors such as p16^{INK4a} or p27^{Kip1} can cause tumor cell cycle arrest (41–43) implying that the pharmacologic inhibition of Cdk activity may be a rational target in cancer therapy.

These and other results have led to an intensive search for small-molecule Cdk inhibitors for cancer therapy (reviewed in refs. 44–46). Flavopiridol, a semisynthetic flavonoid (33) is able to arrest cell cycle in tumor cells in correlation with its ability to directly inhibit Cdk1, Cdk2, Cdk4, Cdk6 (all $IC_{50} \approx 40$ nmol/L), and Cdk7 ($IC_{50} \approx 300$ nmol/L), whereas the IC_{50} values for other protein kinases are all in the mmol/L range (47). Further analysis of flavopiridol in mammalian cells has extended its activities to the induction of apoptosis and angiogenesis or a general inhibition of transcription among others (reviewed in refs. 46, 48, 49). Although far from being an ideal Cdk inhibitor, favopiridol has been extensively used in preclinical and clinical trials with some diverse activity observed in patients with non-Hodgkin lymphoma, renal, prostate, colon, and gastric carcinomas (46, 50). Yet, the molecular heterogeneity of human tumors and lack of patient selection makes it difficult to assess targeted drugs in clinical trials. We have reported in this article the preclinical use of mice genetically modified for cell cycle alterations similar to those occurring in human tumors. Short exposure to flavopiridol results in a significant overall delay of tumor development in Cdk4 and p27^{Kip1} mutant mice, and complete cure of pituitary tumors in 20% of *Cdk4*$^{R/R}$;*p27*$^{+/-}$ mice. This effect correlated with decreased proliferation short after the treatment. This transient effect resulted in a delay in tumor growth as assessed *in vivo* using NMR imaging. The efficiency of the treatment as a cytostatic agent seems to increase in sustained administration protocols, similarly to that described in xenograph models or clinical trials (50).

The current availability of many knock out and knock in mouse strains for most signal transduction pathways should open the way for preclinical trials of targeted drugs. Our results using Cdk4 and p27^{Kip1} mutant mice suggest that genetically engineered mice are a valuable tool to specifically evaluate drugs against the deregulation the INK4-Cdk4 and p27^{Kip1} pathways. Indeed, two of the most frequently altered pathways in human cancer.

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Cooperation between Cdk4 and p27^{kip1} in Tumor Development: A Preclinical Model to Evaluate Cell Cycle Inhibitors with Therapeutic Activity

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