

## Modeling mutations in the G<sub>1</sub> arrest pathway in human gliomas: overexpression of CDK4 but not loss of INK4a-ARF induces hyperploidy in cultured mouse astrocytes

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**Nearly all human gliomas exhibit alterations in one of three genetic loci governing G<sub>1</sub> arrest: INK4a-ARF, CDK4, or RB. To discern the roles of CDK4 amplification and INK4a-ARF loss in gliomagenesis, we compared the behavior of astrocytes lacking a functional INK4a-ARF locus with astrocytes overexpressing CDK4. Either a deficiency of p16<sup>INK4a</sup> and p19<sup>ARF</sup> or an increase in Cdk4 allows cultured astrocytes to grow without senescence. Astrocytes overexpressing CDK4 grow more slowly than INK4a-ARF-deficient astrocytes and convert to a tetraploid state at high efficiency; in contrast, INK4a-ARF-deficient cells remain pseudodiploid, consistent with properties observed in human gliomas with corresponding lesions in these genes.**

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Over half of high-grade human gliomas lack a functional *INK4a-ARF* locus (Jen et al. 1994; Schmidt et al. 1994) and hence can produce neither p16<sup>INK4a</sup> nor p19<sup>ARF</sup>, the two proteins encoded by this locus (Quelle et al. 1995). Most of the remaining gliomas either lack the *RB* gene or demonstrate a 10- to 100-fold amplification of the *CDK4* locus (He et al. 1994, 1995; Ichimura et al. 1996). We are developing animal models for gliomagenesis in hopes of understanding these patterns and discerning the contributions made to tumor formation by each abnormality (Holland et al. 1998). We have taken advantage of two genetic alterations in mice: disruption of the *INK4a-ARF* locus by targeted mutation (Serrano et al. 1996) and astrocyte-specific expression of a transgene encoding TVA, the receptor for subgroup A avian leukosis viruses (ALV) (Holland and Varmus 1998). Production of TVA molecules by these cells makes them susceptible to in-

fection by RCAS vectors carrying coding domains for *CDK4* and other genes (Holland et al. 1998).

We have used this gene transfer system to investigate the effects of *INK4a-ARF* loss and *CDK4* overexpression in astrocyte cell culture and to determine whether similarities exist between the cultured cells and human gliomas with similar abnormalities.

### Results and Discussion

#### *Loss of INK4a-ARF and overexpression of CDK4 both immortalize astrocyte cultures*

To compare the growth properties of *INK4a-ARF*-deficient and *CDK4*-overexpressing astrocytes, we subjected appropriately selected cultures to repeated passage at standard density and counted the number of cells at each passage. Astrocytes overexpressing *CDK4* were prepared by infecting primary brain cultures from *Gtv-a* mice [carrying a *tv-a* transgene under the control of the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter] with an RCAS vector carrying the human *CDK4* cDNA (RCAS-*cdk4*). Cultures of *INK4a-ARF*-deficient astrocytes were prepared by infecting primary brain cell cultures from *Gtv-a* transgenic; *INK4a-ARF*<sup>-/-</sup> mice with an RCAS vector bearing the *puro-R* gene (RCAS-*puro*) (Holland et al. 1998) and selecting for resistance to puromycin. Parallel cultures were prepared by infecting brain cells from *Gtv-a* transgenic mice with RCAS vectors carrying the alkaline phosphatase (*AP*) or the basic fibroblast growth factor (*bFGF*) coding sequences.

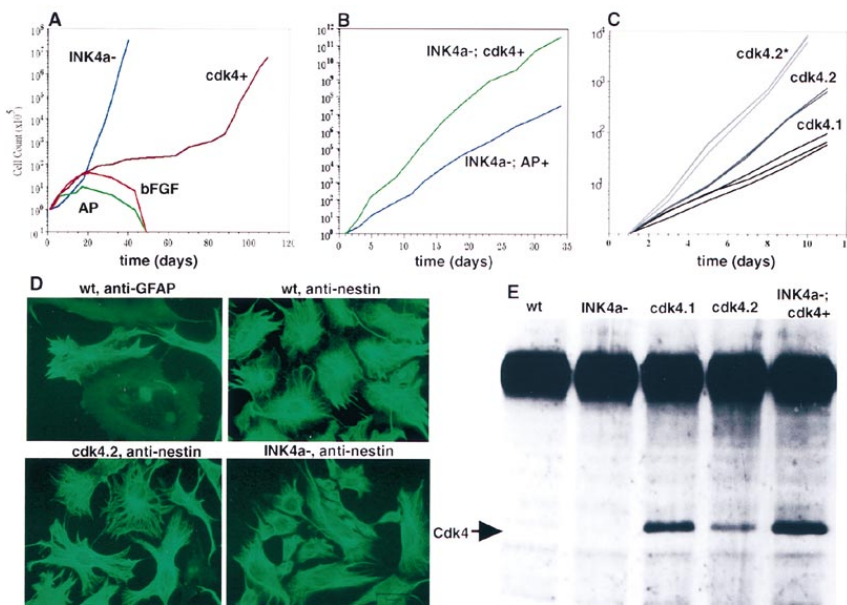
As illustrated in Figure 1A, control astrocytes underwent about three or four cell doublings, gradually entered senescence, and failed to survive beyond ~50 days. Astrocytes infected with RCAS-*bFGF*, which induce proliferation and migration of glial cells in vivo (Holland and Varmus 1998; also see Fig. 4, below), grew to slightly greater numbers than control astrocytes, as expected in view of the known mitogenic effects of bFGF on cultured astrocytes (Hou et al. 1995) but did not survive significantly longer. In marked contrast, *INK4a-ARF*<sup>-/-</sup> astrocytes exhibited no loss of growth potential with passage and could be propagated indefinitely, consistent with the behavior of mouse embryo fibroblasts (MEFs) with lesions in this locus (Alcorta et al. 1996; Nobel et al. 1996) or in ARF alone (Kamijo et al. 1997). Furthermore, astrocytes infected with RCAS-*cdk4* also escaped senescence, implying that both *INK4a-ARF* deficiency and excess *CDK4* allow immortalization of astrocytes. However, the growth rates for these two populations were markedly different; *INK4a-ARF*<sup>-/-</sup> cultures grew at a rapid and constant rate, whereas the *CDK4*-immortalized cultures initially grew slowly and then increased in rate over time.

We demonstrated that the *INK4a-ARF*<sup>-/-</sup> and *CDK4*-immortalized cultures were glia by staining cells with antibodies to GFAP (Bignami and Dahl 1976) and to nestin, an intermediate filament protein expressed in CNS progenitors (Tohyama et al. 1992). *Gtv-a* transgenic pri-

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**Figure 1.** Immortalization of astrocytes by *INK4a-ARF* loss and *cdk4* overexpression. (A) Growth curves comparing *INK4a-ARF*<sup>-/-</sup> astrocytes (*INK4a*<sup>-</sup>) with wild-type *tv-a+* astrocytes infected with *RCAS-CDK4* (*cdk4*<sup>+</sup>), *RCAS-bFGF* (*bFGF*<sup>+</sup>), and *RCAS-AP* (*AP*<sup>+</sup>). (B) Comparison of growth rates between *INK4a-ARF*<sup>-/-</sup> astrocytes infected with *RCAS-AP* (*INK4a*<sup>-</sup>; *AP*<sup>+</sup>) and *RCAS-CDK4* (*INK4a*<sup>-</sup>; *cdk4*<sup>+</sup>). (C) Growth curves comparing two independent *Gtv-a* astrocyte populations infected with *RCAS-CDK4* and maintained in culture for 3 months (*cdk4.1* and *cdk4.2*) relative to the *cdk4.2* population after continuous maintenance in culture for a total of 9 months (*cdk4.2\**). (D) Immunofluorescence staining for GFAP and nestin in *INK4a-ARF*<sup>+/+</sup> astrocytes. Anti-nestin immunofluorescence in *cdk4* immortalized and *INK4a-ARF*<sup>-/-</sup> astrocytes. Magnification, 100 $\times$ . (E) IP-Western blot analysis of cultured cells for expression of Cdk4. The arrow indicates the position of the 34-kD *CDK4* gene product. (wt) Wild-type astrocytes; (*cdk4.1*, *cdk4.2*, *INK4a*<sup>-</sup>; *cdk4*<sup>+</sup>) as above.

many brain cultures infected with *RCAS-puro* initially demonstrated a significant percentage of GFAP<sup>+</sup> cells (Fig. 1D), and virtually all cells expressed nestin as has been observed previously in rat astrocyte cultures (Gallo and Armstrong 1995). To better quantify the population of cells initially infected with *RCAS* vectors, *Gtv-a* transgenic primary brain cultures were infected with *RCAS-GFP*, carrying the gene for green fluorescence protein and analyzed for green fluorescence, GFAP, and nestin. All of the GFP<sup>+</sup> cells were nestin-positive (data not shown). Importantly, 90% of the GFP<sup>+</sup> cells also expressed GFAP, as detected by immunocytochemistry and the remaining 10% of GFP<sup>+</sup> cells not expressing GFAP still displayed an astrocytic morphology. With continued passage, *INK4a-ARF*<sup>-/-</sup> and *CDK4*-immortalized cultures displayed a polygonal, flat morphology characteristic of cultured astrocytes and lost GFAP expression, as observed with astrocytes immortalized by other techniques (Bernard et al. 1994; Frisa et al. 1994). All cells in both populations expressed large amounts of nestin, consistent with a neuroectodermal origin (Fredrikson and McKay 1988). Very large nestin-positive cells were found in both the wild-type and *cdk4*-immortalized populations; in contrast, the *INK4a-ARF*<sup>-/-</sup> cells were primarily small and morphologically immature.

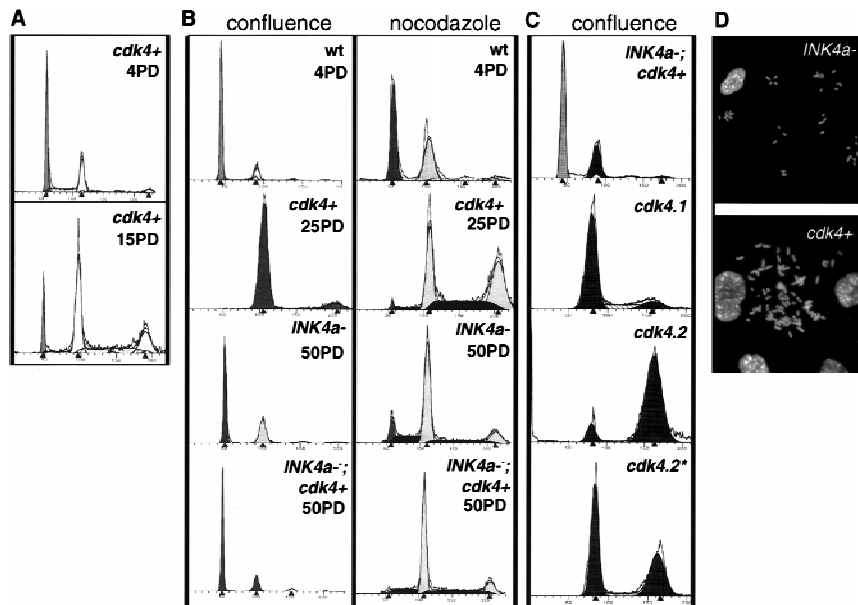
The concentration of Cdk4 in *RCAS-cdk4*-immortal-

ized astrocytes maintained for 3 months in culture was ~5- to 10-fold higher than in wild-type astrocytes (Fig. 1D). These astrocytes grew less rapidly than *INK4a-ARF*<sup>-/-</sup> cells, raising the possibility that excess Cdk4 is a less potent stimulator of growth than a combined deficiency of p19<sup>ARF</sup> and p16<sup>INK4a</sup>. Although we have been unable to examine astrocytes producing higher levels of Cdk4, we have asked whether the growth stimulation provided by excessive Cdk4 would occur in the absence of p19<sup>ARF</sup> and p16<sup>INK4a</sup>. Cultures from brains of *INK4a-ARF*<sup>-/-</sup>; *Gtv-a* transgenic mice were infected with *RCAS-cdk4* and also produced ~10-fold higher than normal levels of Cdk4 (Fig. 1E). These cells grew significantly faster than *INK4a-ARF*<sup>-/-</sup>; *Gtv-a* control infected cells (Fig. 1B). Thus, the growth stimulus provided by excessive Cdk4 would occur in the absence of p19<sup>ARF</sup> and p16<sup>INK4a</sup> products.

#### *CDK4 overexpression induces hyperploidy that is INK4a-ARF dependent*

Tumor cells, including glioma cells, often contain an abnormal number of chromosomes (Bigner and Mark 1984), and hyperploidy occurs when mechanisms for control of cell cycle progression have been disrupted (Tahanda et al. 1995). Therefore, we asked whether euploidy is maintained in cultured astrocytes with a deficiency of *INK4a-ARF* or an excess of Cdk4, and whether any abnormalities in ploidy are correlated with specific genetic mutations, as reported in human gliomas (van Meyel et al. 1994). Flow cytometry was used to assess DNA content in astrocytes with different passage histories after maintenance at confluence or after addition of nocodazole.

By these measures, six of six astrocyte cultures independently infected with *RCAS-cdk4* were mostly converted to tetraploid status within 15–20 population doublings (PD), at a rate approaching 10% of cells per generation (Fig. 2A). After 25 PDs, virtually all *CDK4*-expressing cells had twice the normal amount of DNA at confluence, and nearly half of the nocodazole-treated cells were arrested in G<sub>2</sub>/M with an 8N DNA content (Fig. 2B). In sharp contrast, *INK4a-ARF*-deficient cells were very similar to wild-type astrocytes, even after >50 PDs. In nocodazole, a higher proportion of *INK4a-ARF*-deficient cells than wild-type cells were arrested in G<sub>2</sub>/M, but <10% of the cells had an 8N DNA content (Fig. 2B, right). These findings were confirmed by direct inspection of metaphase chromosomes (Fig. 2D); chromosome numbers in metaphase spreads from *INK4a-ARF*-



**Figure 2.** RCAS-*cdk4*-infected astrocytes shift to tetraploidy. Flow cytometry analysis of the indicated cultures after propidium iodide staining. (A) CDK4-immortalized cells analyzed after 4 and 15 population doublings (PDs). (B) Cultures analyzed at confluence ( $G_1$  arrest) after the indicated number of PDs and after treatment for 16 hr with 0.12  $\mu\text{g}/\text{ml}$  nocodazole (right). (C) Flow cytometry of cell cultures at confluence illustrating the pseudodiploid RCAS-*cdk4*-infected *INK4a-ARF*<sup>-/-</sup>; *Gtv-a* astrocytes maintained for 9 months in culture compared with the hyperploid *cdk4.1*, *cdk4.2*, and *cdk4.2\** populations described above. (D) Metaphase spreads of diploid *INK4a-ARF*<sup>-/-</sup> and tetraploid CDK4-immortalized cells as indicated.

deficient cells were ~40 and thus diploid or pseudodiploid, whereas CDK4-overexpressing cells had approximately twice the normal number of chromosomes.

The number of recognized genetic alterations capable of immortalizing cells in culture is relatively small; and of those, only loss of p53 function and hyperproduction of Myc protein have been reported previously to induce hyperploidy. The mechanism by which elevated levels of Cdk4 induce hyperploidy is unknown but in some way must result from endoduplication of chromosomes and aberrant cell cycle arrest in  $G_2$ .

We next examined the DNA content of *INK4a-ARF*<sup>-/-</sup>; *Gtv-a* astrocytes infected with RCAS-*cdk4* and producing at least 10-fold more Cdk4 than normal cells (Fig. 1E). Surprisingly, these cells maintained pseudodiploidy, implying a requirement for one or both of the products of the *INK4a-ARF* locus for induction of inappropriate rounds of DNA replication by Cdk4. p19<sup>ARF</sup> functions by binding to Mdm2 and thereby inactivating p53 (Kamijo et al. 1997; Pomerantz et al. 1998; Zhang et al. 1998) and is involved in both  $G_1$  and  $G_2$  arrest (Quelle et al. 1997). Therefore, it may be a more likely candidate to promote Cdk4-induced hyperploidy than p16<sup>INK4a</sup>, which is known only to inhibit Cdk4 and block passage from  $G_1$  to S phase. Overexpression of CDK4 in astrocytes derived from mice with targeted mutations specific for p16<sup>INK4a</sup> or p19<sup>ARF</sup> would help identify the *INK4a-ARF* product required for Cdk4-induced hyperploidy.

Curiously, although mutations in p53 lead to aneu-

ploidy in culture, a mutation that specifically eliminates the production of p19<sup>ARF</sup> results in pseudodiploid immortalized cells (Kamijo et al. 1997). It is not known whether the shift in ploidy seen in p53<sup>-/-</sup> cells is dependent on the presence of wild-type p19<sup>ARF</sup> or p16<sup>INK4a</sup> as appears to be the case for the ploidy shift due to *cdk4* overexpression.

#### *Cdk4*-induced immortalization and hyperploidy can occur independent of p53 mutations

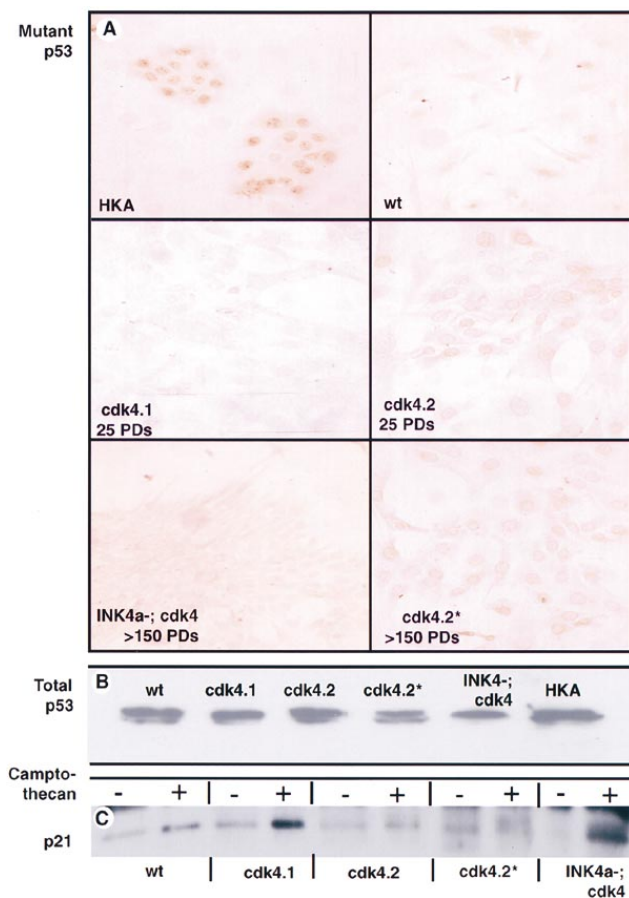
Mutations in p53 are associated with loss of growth control and chromosomal instability (Levine 1993). Thus, secondary mutations of p53 in astrocytes infected with RCAS-*cdk4* could be responsible for the properties described above. To address this possibility we analyzed two independent populations of RCAS-*cdk4*-infected *Gtv-a* astrocytes that had been maintained in culture for 3 months. Both populations (*cdk4.1* and *cdk4.2*) showed elevated levels of Cdk4 by Western analysis (Fig. 1D). Flow cy-

tometry of these two populations (performed on day 6 of the experiment shown in Fig. 3) demonstrated that *cdk4.1* cells are mostly tetraploid and *cdk4.2* cells mostly octaploid.

We judged p53 status using three criteria: immunohistochemical staining with antibodies specific for the mutant conformation of p53; the level of total p53 by Western blot analysis; and the induction of p21 after DNA damage. By all three criteria the *cdk4.1* population showed no evidence of mutant p53 (Fig. 3). These data imply that in the *cdk4.1* culture, immortalization and hyperploidy occurred in the presence of wild-type p53 protein and normal p53 function.

The *cdk4.2* culture, in contrast, stained positively with the antibody for the mutant p53 and failed to induce p21 after camptothecin treatment. The *cdk4.2* population was maintained in culture for an additional 6 months (*cdk4.2\**) and analyzed for ploidy, growth rate, and p53 status. The *cdk4.2\** cells demonstrated increased growth rate relative to that seen at 3 months (Fig. 1C), maintained evidence of mutant p53 by immunocytochemical criteria, and lacked p21 induction after camptothecin treatment. Surprisingly, flow cytometry showed that the population shifted from being mainly octaploid at 3 months to mainly tetraploid at 9 months. Presumably, the increased growth rate observed between the *cdk4.2* population after 6 additional months in culture reflects the further occurrence of mutations, epigenetic events, or both.





**Figure 3.** Mutations in *p53* can, but do not necessarily, arise in *CDK4*-immortalized populations. (A) Immunoperoxidase staining of cell cultures using a monoclonal antibody to the mutant conformation of *p53*. Wild type, *cdk4.1*, *cdk4.2*, *cdk4.2\**, and *INK4a-; cdk4\** are as in Fig. 1, (HKA) human keratinocyte cell line known to harbor a *p53* mutation. (B) Western analysis for *p53* in the indicated cultures. (C) Western analysis for *p21* in cultured cells with and without camptothecin-induced DNA damage.

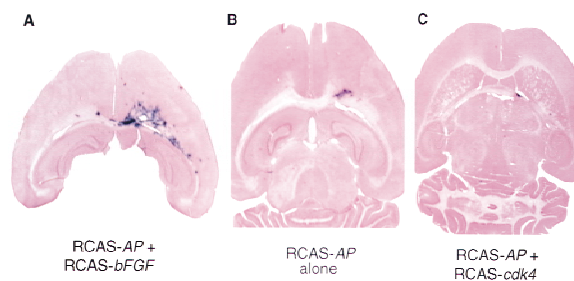
The existence of a *CDK4*-immortalized and hyperploidy culture with wild-type *p53* function (*cdk4.1*) implies that *CDK4*-overexpressing astrocytes do not require mutations in *p53* to achieve extended proliferation and genomic instability. The extended life span of the *cdk4.1* population could be formally explained by a secondary mutation in *INK4a-ARF* or *ARF* alone. However, this is unlikely, as *INK4a-ARF*; *CDK4*-overexpressing astrocytes are pseudodiploid and rapidly proliferating, whereas the *cdk4.1* population is polyploid and grows more slowly. Furthermore, Southern analysis of the *cdk4.1* population with the *p19<sup>ARF</sup>* cDNA did not demonstrate deletions or alterations in the genomic structure of the *INK4a-ARF* locus (data not shown). These data imply that additional mutations in *p53* or *INK4a-ARF* are not required for immortalization of *CDK4*-overexpressing astrocytes. In contrast, immortalization of MEFs by *myc* overexpression selects for those that lose either *p53* or *p19<sup>ARF</sup>* function (Zindy et al. 1998) and

MEF cultures selected for spontaneous immortalization develop mutations in *p53* or *ARF* (Kamijo et al. 1997). Although mutations in *p53* have long been known to result in both immortalization of cells in culture and hyperploidy, the pathways leading to these effects are not completely understood. One of the effects of *p53* is to increase the concentration of *p21*, which, in turn, inhibits *Cdk4*. Therefore, loss of *p53* function might be expected to result in higher *Cdk4* activity. It would be valuable to know whether the immortalization and ploidy shifts seen in *p53*-deficient cells are a result of, or dependent on, inappropriately elevated *Cdk4* activity.

#### Overexpression of *cdk4* by astrocytes in vivo does not induce proliferation

To gauge the physiological significance of *Cdk4* overproduction in astrocytes in vivo, we infected newborn *Gtv-a* transgenic mice with *RCAS-cdk4*. With this method, cells lining the injection track are coinfecting with *RCAS-AP* to monitor cells (Holland and Varmus 1998). *AP<sup>+</sup>* cells are no more numerous or widely dispersed after coinfection with a mixture of *RCAS-AP* and *RCAS-cdk4* than after infection with *RCAS-AP* alone (Fig. 4B,C). In contrast, as described previously (Holland and Varmus 1998), *AP<sup>+</sup>* cells are highly abundant and spread over a large expanse of the brain after coinfection with a mixture of *RCAS-AP* and *RCAS-bFGF* (Fig. 4A). Thus, by itself, excessive levels of *Cdk4* do not appear to perturb the proliferative or migratory behavior of astrocytes in vivo.

The absence of gliomas in *INK4a-ARF*-deficient mice (Serrano et al. 1996) suggests that a lack of both *p16<sup>INK4a</sup>* and *p19<sup>ARF</sup>* is insufficient to produce grossly abnormal growth of glial cells in vivo; this is similar to our results with gene transfer of *cdk4* to astrocytes in *Gtv-a* transgenic mice (Fig. 4). However, either *INK4a-ARF* loss or *CDK4* overexpression can immortalize astrocytes in vitro; thus, additional limitations on proliferation may be mediated by unknown mechanisms in vivo. Of note, cultures of both *INK4a-ARF<sup>-/-</sup>* and *CDK4*-immortalized cells arrest in *G<sub>1</sub>* upon reaching confluence, implying the



**Figure 4.** Glial-specific *CDK4* gene transfer does not result in proliferation in vivo. An equal mixture of cells producing *RCAS-AP* and *RCAS-bFGF* (A), *RCAS-AP* alone (B), or *RCAS-AP* and *RCAS-cdk4* (C) was injected into the right frontal lobe of *Gtv-a* mice. The mice were sacrificed and brains (40- $\mu$  sections) analyzed for *AP* activity at 10 weeks of age and counterstained.

existence of an intact G<sub>1</sub>-arrest pathway in these cells. Identification of factors and pathways causing growth arrest of either *INK4a-ARF*<sup>-/-</sup> or *CDK4*-immortalized astrocytes in culture may help elucidate the mechanism for arrest of these cells in vivo.

Recently, we have reported the utility of transgenes expressing *tv-a* in the glial lineage in intact animals (Holland and Varmus 1998; Holland et al. 1998). Here we demonstrate that primary cell lines from such *tv-a* transgenic animals can be used to perform high efficiency gene transfer to defined cells within a mixture of cell types. Selection for specific, infectable cell types is possible if the population is initially infected with RCAS-*puro* and subsequently grown in puromycin. The ability to manipulate populations of primary astrocytes genetically in culture has allowed us to study the effects of individual alterations in the G<sub>1</sub> arrest pathways, something not possible in established cell lines in which the endogenous G<sub>1</sub> arrest pathway has already been altered.

In Holland et al. (1998) we describe the contributions that excess Cdk4 or loss of the *INK4a-ARF* gene products make to gliomagenesis. We find that a constitutively active, mutant *EGFR* is insufficient to induce gliomas in mice, but mutant *EGFR* can induce glioma formation either in *INK4a-ARF*<sup>-/-</sup> mice or, less often, in combination with excess Cdk4. Furthermore, mutant *EGFR* does not induce gliomas in *p53*-deficient mice unless *CDK4* is overexpressed. Our results demonstrate that *INK4a-ARF* loss and *CDK4* overexpression not only immortalize astrocytes in culture, as shown here, but are also important components of gliomagenesis in mice. The fact that *p53* mutations arise in some *CDK4*-immortalized astrocyte cultures implies that *p53* loss can provide a growth advantage to these cells. The results in Holland et al. (1998) suggest that *CDK4* overexpression and *p53* loss also cooperate in gliomagenesis. These apparent interactions between components of the cell cycle arrest pathways in mice and in cultured mouse astrocytes resemble the genetic abnormalities found in human gliomas. Most notably, results in the two species illustrate the importance of disrupting the p16<sup>INK4a</sup>-Cdk4-Rb pathway, the nonequivalence of mutations in the pathways that govern G<sub>1</sub>-S transition, and the apparent synergy between *CDK4* overexpression and *p53* loss. Taken as a whole, these observations indicate that behavior of genetically defined primary astrocyte cultures reflects many aspects of gliomagenesis both in mice and man.

## Materials and methods

### Transgenes and viral vectors

Construction of the *Gtv-a* transgene and RCAS-*AP* and RCAS-*bFGF* have been described (Holland and Varmus 1998). The *Gtv-a* mouse line was originally generated from an FVB/N crossed with a C57B6 × BALB/c F<sub>1</sub>. The *Gtv-a* founder was then bred to an FVB/N to generate F<sub>1</sub> progeny that have subsequently been interbred to maintain the transgenic line. RCAS-*puro* was obtained from Steve Hughes (National Cancer Institute). RCAS-*cdk4* was constructed by *Clal* digestion of RCAS-*puro* to remove the *Escherichia coli* puromycin resistance gene and replacement with a *Bst*BI-*Clal* fragment from *pcdk4.1* (gift from Robert Sikorski), which contains the complete human *CDK4* cDNA (Matsushime et al. 1992). RCAS-*GFP* was a gift of Connie Cepko (Harvard University, Cambridge, MA).

### Cell culture

Primary brain cell cultures from newborn transgenic mice were obtained by mechanical dissociation of the whole brain, followed by digestion with 0.25% trypsin for 15 min at 37°C. Large debris was allowed to settle, and single cells were plated and grown in DMEM with 10% FCS (GIBCO BRL). DF-1 cells (gift from D. Foster; Schaefer-Klein et al. 1998) were grown in DMEM with 5% FCS, 5% calf serum, 1% chicken serum, and 10% tryptose phosphate broth (GIBCO BRL).

### Infection with RCAS vectors

The supernatant from DF-1 cells infected with and producing RCAS vectors was filtered through a 0.45-μ filter and plated directly onto primary brain cells cultures from *Gtv-a* mice. *INK4a-ARF*<sup>-/-</sup>; *Gtv-a* cultures were generated from the F<sub>2</sub> progeny of *Gtv-a* mated with mice having targeted deletions of *INK4a-ARF* (gift of Ron DePinho, Harvard Medical School, Boston, MA). These primary brain cultures were infected with filtered medium from RCAS-*puro*-producing cells and then selected in 4 μg/ml puromycin. To infect cells in *Gtv-a* transgenic mice, DF-1 cells infected with RCAS vectors were harvested by trypsin digestion and pelleted by centrifugation, the cell pellets were resuspended in ~50 μl of medium, and placed on ice. Using a 10-μl gas-tight Hamilton syringe, a single intracranial injection of 1 μl (containing 10<sup>4</sup> cells) was made in the right frontal region, just anterior to the striatum, with the tip of the needle just touching the skull base.

### Brain sectioning and staining

Animals were sacrificed at 10 weeks of age, the brains fixed in 4% formaldehyde, 0.4% glutaraldehyde, 1× PBS for 36 hr, and dehydrated in 20% sucrose, 2% glycerol, and 1× PBS. Frozen sections (40 μm) were obtained using a sledge microtome (Zeiss) and stained in solution for alkaline phosphatase activity using 5-bromo-4-chloro-indolyl-phosphate and 4-nitro-blue-tetrazolium-chloride (Boehringer), after treatment at 65°C (pH 9.5) for 30 min to remove endogenous alkaline phosphatase activity. The sections were then mounted on glass slides and counterstained with hematoxylin and eosin.

### Flow cytometry

Cultures were either grown to confluence and maintained for 24 hr or treated with 0.12 μg/ml nocodazole (Sigma) for 16 hr. Cells (5 × 10<sup>5</sup>) were harvested by trypsin digestion, centrifuged, dispersed in 500 μl of propidium iodide solution (Electa), incubated for 20 min at 37°C, and analyzed on a Beckman FaxScan using ModFit LT software (Verity).

### Immunofluorescence and immunohistochemistry

Cell cultures used for immunostaining were grown on glass coverslips precoated with 0.1 mg/ml poly-D-ornithine (Sigma). For staining with anti-GFAP antibodies, cells were fixed in 4% paraformaldehyde (pH 7.4 in PBS) for 15 min, permeabilized in 95% ethanol/5% acetic acid for 10 min, and incubated with 1:300 diluted rabbit anti-human GFAP antibody (Chemicon) for 1 hr. After incubation with fluorescein- or rhodamine-conjugated goat anti-rabbit (GAR; Cappel-Organon Teknika) for 45 min, cells were washed extensively in PBS and mounted in Vectashield (Vector Laboratories). For staining with anti-nestin antibody, cells were fixed and permeabilized as described above and incubated overnight at 4°C with an anti-nestin polyclonal rabbit antibody (gift from Ron McKay; Tohyama et al. 1992) (1:1000; in 1% fetal bovine serum + 0.5% bovine serum albumin). After incubation with fluorescein- or rhodamine-conjugated GAR for 45 min at room temperature, cells were washed extensively in PBS and mounted in Vectashield. The immunofluorescence micrographs presented are representative of two to three experiments and were taken on a Zeiss Axiophot fluorescence microscope (40× Neo-fluar objectives).

For detecting mutant p53, cells were initially fixed with 100% methanol and incubated with Tris-buffered saline (pH 8.0), 0.1% Tween 20, (TBST) 5% dried milk, and 1% goat serum (TBST). A mouse monoclonal antibody recognizing mutant p53 in nondenaturing conditions (Ab-3, Oncogene Science) was incubated at a 1:200 concentration in TBST for 1 hr at room temperature. The cells were washed with PBS and the antibody detected with a biotin-conjugated anti-mouse antibody and avidin-horse-radish peroxidase (ABC kit, Vector Labs).

### Metaphase spread

Cells were treated with 0.02 mg/ml Colcemid (Sigma) for 6 hr and harvested by trypsin digestion and centrifugation. The cells were resus-

pended in 0.06 M KCl, fixed in methanol/acetic acid (3:1), dropped onto glass slides, stained with 1 µg/ml DAPI (Polyscience), and visualized by fluorescence microscopy.

#### Western blot analysis

Total cell proteins (0.6 mg) isolated in RIPA buffer were precipitated overnight at 4°C with 0.4 µg of anti-Cdk4 antibody (Santa Cruz) using protein A-Sepharose (Sigma). The products were separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated with the same anti-Cdk4 antibody, washed extensively in TBST and incubated with a HRP-conjugated GAR antibody (Boehringer) and detected with ECL.

p21 was induced by treating cultures at 70% confluence with 300 nM camptothecin (Sigma) for 24 hr. Total cellular protein was isolated in RIPA buffer, separated by SDS-PAGE transferred to nitrocellulose, and probed with p21 monoclonal antibodies (Ab-5, Calbiochem). The antibodies were visualized using HRP-conjugated, anti-mouse antibody (Boehringer), and ECL. For the p53 Western blot, total protein from untreated cultured cells was isolated and analyzed as for p21, using an anti-p53 antibody that recognizes both mutant and wild-type p53 proteins after denaturation under SDS-PAGE (Ab-3, Oncogene Science).

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