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Extensive contribution of *Rb*-deficient cells to adult chimeric mice with limited histopathological consequences

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Homozygosity for a mutation in the *Rb* tumor suppressor gene causes mid-gestation embryonic lethality in the mouse. Using a two-step targeting protocol, we have constructed *Rb* homozygous mutant mouse embryonic stem cells and used them to create chimeric animals partially composed of *Rb*-deficient cells. Analysis of these chimeras demonstrates widespread contribution of the mutant cells to adult tissues, including the retina and mature erythrocytes. Despite the presence of large numbers of *Rb*-deficient cells in most tissues of these mice, they are remarkably normal but do exhibit certain histological defects including cataracts, hyperplasia of the adrenal medulla, and enlarged cells in the cerebellum and the liver. Like animals heterozygous for the *Rb* mutation, the chimeras develop tumors of the intermediate lobe of the pituitary, and the rate of pituitary tumorigenesis is greatly accelerated.

Key words: embryonic lethality/mouse/*Rb* tumor suppressor gene/tumorigenesis

Introduction

The development of many human tumors is associated with mutations in tumor suppressor genes (Knudson, 1993). One of the best studied of these genes is the retinoblastoma susceptibility gene (*RB*). In humans, inheritance of a defective allele of the *RB* gene strongly predisposes to pediatric eye cancer, retinoblastoma (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). The gene is also mutated during the development of sporadic retinoblastomas as well as osteosarcomas, soft tissue sarcomas, and carcinomas of the breast, lung and bladder (Friend *et al.*, 1986, 1987; Fung *et al.*, 1987; Lee *et al.*, 1987; Harbour *et al.*, 1988; E.Y.-H.P.Lee *et al.*, 1988; T'Ang *et al.*, 1988; Yokota *et al.*, 1988; Varley *et al.*, 1989; Bookstein *et al.*, 1990b; Hensel *et al.*, 1990). Introduction of wild-type *RB* into various tumor cell lines which have lost endogenous *RB* function can inhibit tumorigenicity and cell cycle progression or induce cell senescence (Huang *et al.*, 1988; Bookstein *et al.*, 1990b; Qin *et al.*, 1992). These results provide direct evidence that this gene can act to limit cell proliferation and that its loss contributes to the decontrolled growth associated with these tumor cell types.

To study the function of *RB* in more detail, we and others have created mouse strains carrying germline mutations in its murine homolog, *Rb* (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Analysis of animals heterozygous for the targeted disruption revealed no evidence of predisposition to retinoblastoma. However, the heterozygous animals were highly susceptible to adenocarcinoma of the pituitary, with ~90% of the animals developing this tumor by 1 year of age (B.O.Williams, L.Remington, R.T.Bronson and T.Jacks, unpublished data). In addition, heterozygotes are susceptible to medullary thyroid carcinoma, with 70% of animals developing this tumor by 1 year of age (B.O.Williams, L.Remington, R.T.Bronson and T.Jacks, unpublished data). As with human retinoblastoma (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987), pituitary and thyroid tumorigenesis in these mice was accompanied by the mutation of the wild-type allele of *Rb* (Jacks *et al.*, 1992; B.O.Williams, L.Remington, R.T.Bronson and T.Jacks, unpublished data).

The *Rb* mutant mice also provided the opportunity to study the role of this tumor suppressor gene in normal development. Homozygosity for the *Rb* mutation was shown to cause embryonic lethality between days 13.5 and 15.5 of gestation (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Death was associated with defects in the production of mature erythrocytes in the liver and widespread cell death in the central nervous system. The development of other tissues, including the retina, appeared to occur normally in the *Rb*-deficient embryos.

The analysis of homozygous mutant embryos has suggested a requirement for *Rb* in normal development of at least erythroid and neuronal tissues. Due to the attendant lethality, however, it was not possible to determine what effects, if any, the absence of *Rb* function would have on normal development or neoplasia at later stages of gestation or postnatally. To address these issues, we have created embryonic stem (ES) cells that are homozygous for the *Rb* mutation and injected them into wild-type blastocysts, yielding chimeric animals. Due to the contribution of the wild-type cells from the host embryo, many of these chimeras escape the mid-gestation lethality caused by germline *Rb* homozygosity and develop to birth. This has allowed us to assess the pathological and tumorigenic effects of the widespread presence of *Rb*-deficient cells in later stages of development and in adult life.

Results

Construction of homozygous mutant ES cells and generation of chimeric animals

Rb homozygous mutant (*Rb*-DKO) cells were created using a two-step targeting protocol (te Riele *et al.*, 1990).

First, heterozygous D3 ES cells (Gossler *et al.*, 1986) (derived from strain 129/sv) were constructed using an *Rb* targeting vector carrying a neomycin resistance gene (*neo*) (Jacks *et al.*, 1992). The surviving wild-type *Rb* allele present in a single heterozygous ES cell clone was then re-targeted using a second vector, identical to the first except that a hygromycin resistance gene (*hygro*) was substituted for the *neo* gene (Figure 1A). *Rb*-DKO cells were identified by Southern blotting following selection in G418, hygromycin and gancyclovir.

As shown in Figure 1B, resulting homozygous mutant cells lack the band corresponding to the wild-type *Rb* allele and contain instead an additional fragment representing the *Rb-hygro* mutation. Of 173 ES cell clones resistant to the three drugs, six were found to be homozygous for the *Rb* mutation, with the herpes simplex virus thymidine kinase (*HSV-TK*) counterselection (Mansour *et al.*, 1988) providing an ~6-fold enrichment for correctly targeted clones. Thus, the efficiency of targeting the second *Rb* allele was approximately one in 173 hygromycin-resistant cell clones, similar to that observed for the first round of gene targeting (Jacks *et al.*, 1992). Consistent with earlier analysis of embryos homozygous for the original Rb^{x3t} mutation (Jacks *et al.*, 1992), the *Rb*-DKO ES cells were also found to lack detectable *Rb*-encoded protein (pRb) by Western blotting (data not shown).

Three independently derived *Rb*-DKO ES cell clones were then injected into C57Bl/6 blastocysts to create chimeric animals. For comparison, several chimeras were also generated using the parental heterozygous ES cell clone. Given the lethality caused by germline homozygosity for the *Rb* mutation, we assumed that injection of the standard 12–15 ES cells per blastocyst (Bradley, 1987) might lead to embryonic lethality and reduced the number of *Rb*-DKO cells injected per blastocyst to approximately eight. A systematic analysis of the effects of injection of different numbers of mutant cells was not performed.

Contribution of Rb-DKO cells to adult tissues

The contribution of the *Rb*-deficient cells to chimeric animals could be estimated from the extent of agouti coat color (the product of the *Rb*-deficient 129/SV ES cells) and was found to range from ~30 to 60%. In addition, we wished to determine the percent contribution of mutant cells to the internal tissues of these chimeras. The *Rb*-DKO ES cells are homozygous for the *a* allele of glucose phosphate isomerase (*GPI-1s*^{a}, encoding GPI-1A) while cells of the C57Bl/6 host embryo are homozygous for *GPI-1s*^{b} (encoding GPI-1B) (Lyon and Searle, 1990). Thus, by using cellulose acetate electrophoresis and staining for the specific enzyme activity of the two isoforms (Bradley, 1987), we could determine the relative contribution of the *Rb* −/− cells to individual tissues (Figure 2A). More than 35 tissues from 14 *Rb*-DKO chimeras and five controls were examined using this assay.

As summarized in Figure 2B, we observed significant contribution of the *Rb*-DKO cells to all adult tissues examined, including liver, thymus, spleen, kidney and muscle. These data indicate that *Rb* function is not required for the differentiation of cells in many adult tissues. However, since the GPI assay only measures the percent contribution to the tissue as a whole, it is not possible to

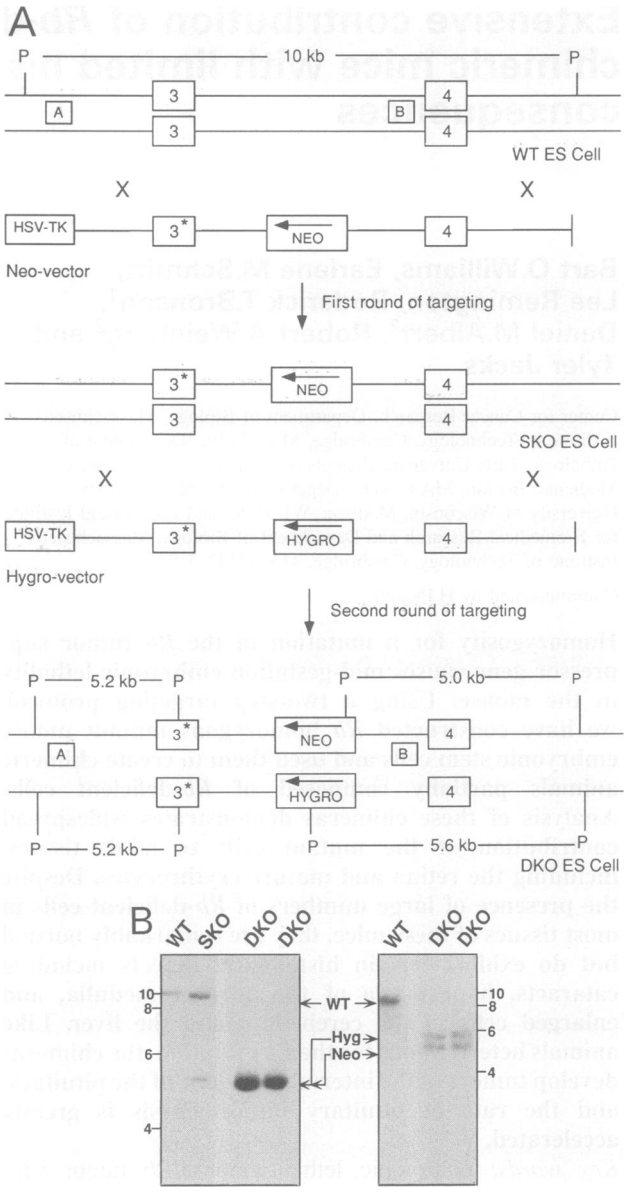

Fig. 1. Construction of *Rb*-deficient cells. (**A**) Gene targeting strategy to create *Rb*-DKO ES cells. One allele of the *Rb* gene was mutated in wild-type (WT) D3 ES cells (Gossler *et al.*, 1986) in the first round of targeting using a vector carrying a mutation in the third exon (designated 3*) and a neomycin resistance gene (*neo*) in the third intron. The exon 3 mutation creates two tandem termination codons and a novel *Pst*I (P) recognition site (Jacks *et al.*, 1992). The remaining wild-type allele in the heterozygous (or SKO) ES cell was then subjected to a second round of gene targeting with a vector identical to the first except that the hygromycin resistance gene (*hygro*) replaced *neo* (*hygro* targeting vector), yielding the *Rb*-deficient (or DKO) ES cells. Both targeting vectors contain the herpes simplex virus thymidine kinase gene (*HSV-TK*) to provide counterselection (Mansour *et al.*, 1988). As indicated by arrows, the drug selection genes are in the opposite transcriptional orientation from *Rb*. The lengths of homology to *Rb* in both targeting vectors are 1.1 kb upstream of the drug resistance gene and 8.5 kb downstream (Jacks *et al.*, 1992). Clones were screened by Southern blotting of *Pst*I-digested genomic DNA using probes A and B (boxed). Lengths of relevant *Pst*I restriction fragments are indicated. (**B**) Southern blots of *Rb*-mutant ES cells. *Pst*I-digested genomic DNA isolated from wild-type ES cells, the parental SKO ES cell clone and several DKO ES cell clones was hybridized to probes A (left) and B (right) shown in panel A. Positions of the wild-type (WT), *neo* and *hygro* mutant alleles are shown along with the positions of molecular weight standards (in kb).

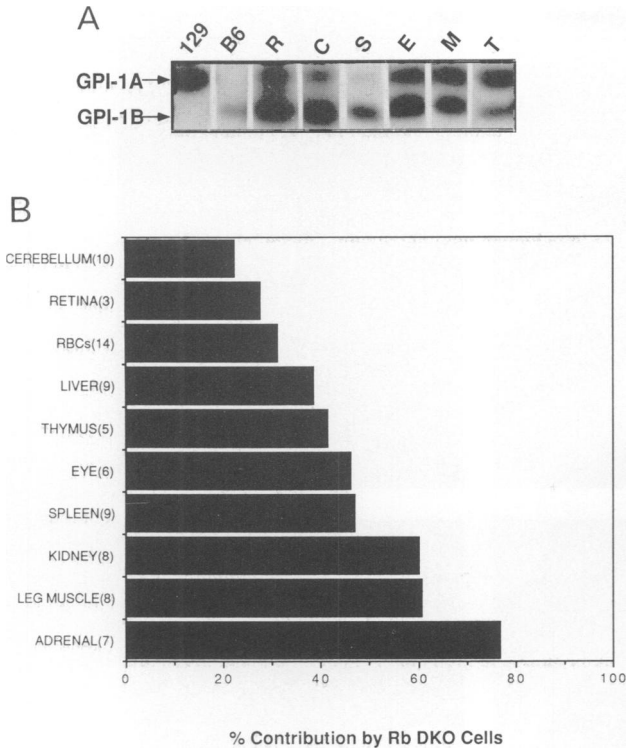

Fig. 2. GPI isoenzyme analysis. (**A**) GPI activity assay. Lysates from several tissues isolated from *Rb*-DKO chimeras were separated by cellulose acetate electrophoresis and stained for GPI activity as described in Materials and methods. Tissues shown are retina (R), cerebellum (C), spinal cord (S), red blood cells (E), bone marrow (M) and tail (T). *Rb*-DKO cells (derived from strain 129/Sv) express the GPI-1A isoform, while host C57BL/6 cells produce GPI-1B. The migration of the two isoforms is shown in control lanes of 129/Sv tissue (129) and C57Bl/6 tissue (B6) and is indicated on the left. (**B**) Quantification of tissue contribution of *Rb*-DKO cells. Graph shows the average contribution (by percentage) of *Rb*-deficient cells to representative tissues isolated from *Rb*-DKO chimeras. The number of animals from which each tissue was examined is indicated in parentheses. The lowest and highest values of *Rb* −/− contribution to tissues in individual chimeras were the following: cerebellum (low of 2.5%, high of 54.4%), retina (22.9%, 30.2%), red blood cells (1.8%, 64.8%), liver (4.3%, 83.9%), thymus (22.8%, 60.3%), eye (33.2%, 61.1%), spleen (13.7%, 66.3%), kidney (49.9%, 77.9%), leg muscle (37.5%, 78.7%) and adrenal (61.2%, 97.7%). A list of the 36 different tissues tested in total is provided in Materials and methods.

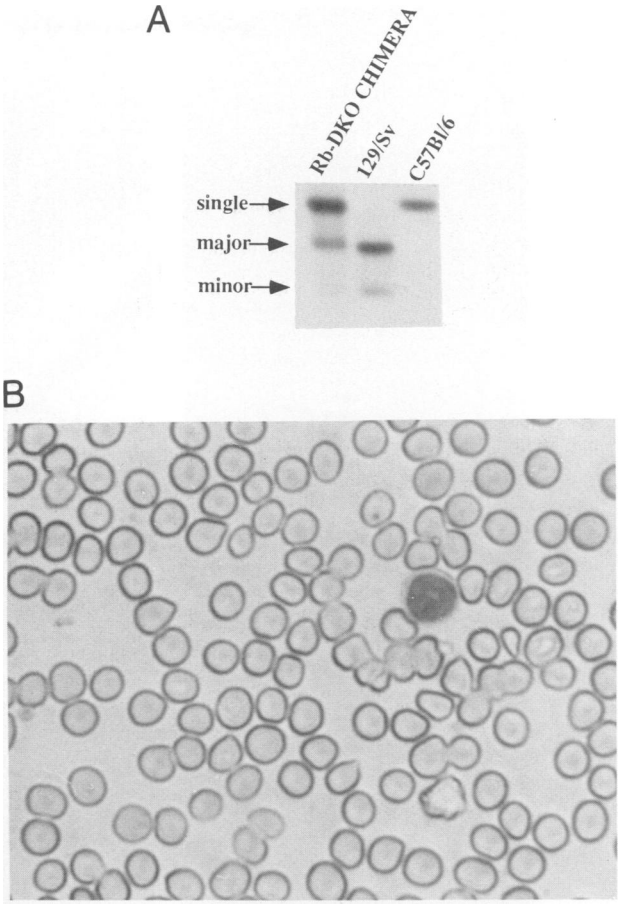

Fig. 3. Normal erythropoiesis in *Rb*-DKO chimeras. (**A**) β-globin isoforms. Blood proteins from an *Rb*-DKO chimera and 129/Sv and C57Bl/6J controls were separated by cellulose acetate electrophoresis and stained as indicated in Materials and methods. The presence of *Rb*-deficient erythroid cells in the *Rb*-DKO chimeras is verified by the production of the 129/Sv-specific Hb major and Hb minor species; Hb single (produced by strain C57Bl/6 red blood cells) is also evident in the chimeras. (**B**) Normal erythrocytes. A typical blood smear (stained with Wright−Giemsa) from an *Rb*-DKO chimera shows normal red blood cells and a lack of nucleated erythrocytes. Blood smears from control chimeras generated from ES cells heterozygous for the *Rb* mutation were indistinguishable from those derived from *Rb*-DKO chimeras (data not shown).

conclude that all cell types within that tissue can differentiate in the absence of *Rb* function.

As reported previously, embryos homozygous for an *Rb* mutation die at 13.5–14.5 days *in utero* with defects in hematopoiesis and neurogenesis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Thus, it was of particular interest to examine the ability of *Rb*-deficient cells to contribute to these tissues in the *Rb*-DKO chimeras. As shown in Figure 2B, GPI isoenzyme analysis clearly demonstrates significant contribution by the *Rb*-deficient cells to at least some portions of the adult brain, spinal cord and red blood cell (RBC) compartment. The percentage of *Rb*-deficient cells in the cerebellum (and other central nervous system tissues) was consistently among the lowest of all the tissues examined (Figure 2B, data not shown). However, this pattern was also observed in chimeras made from ES cells heterozygous for the *Rb* mutation (data not shown), and, therefore, presumably reflects the relative ability of the ES cell-derived donor cells to compete with the cells of host embryo during organogenesis.

The rather extensive contribution of the *Rb*-deficient cells to the RBC compartment was unexpected in light of the observed inhibition of erythropoiesis in *Rb* homozygous mutant embryos (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). In order to confirm that the GPI-1A signal measured from the purified RBC fraction was derived from *Rb*-deficient erythrocytes (rather than some contaminating cell type), we assayed for the presence of the Hb major and Hb minor forms of β-globin produced by the Hbb^{d} haplotype of strain 129/sv (Whitney, 1977). As shown in Figure 3A, erythrocytes isolated from strain C57BL/6 animals produce Hb single (encoded by the Hbb^{b} haplotype), which is separable from the 129/sv-specific Hb major and Hb minor species. The RBC fraction isolated from *Rb*-DKO chimeras clearly contains all three β-globin forms (Figure 3A), indicating the presence of *Rb*-deficient hemoglobinized cells. Peripheral blood

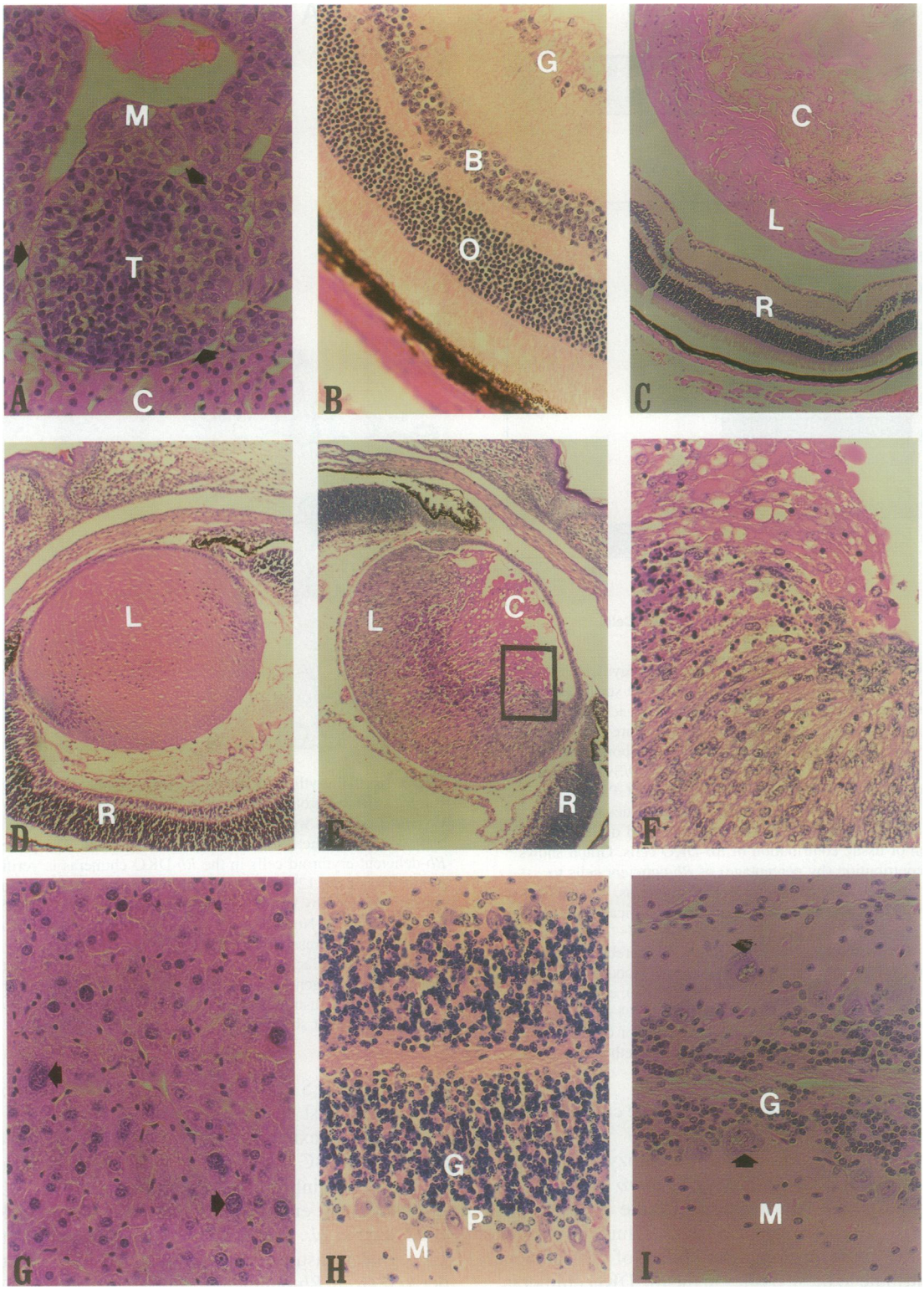

Fig. 4. Histopathology. (**A**) Hyperplastic nodule (T) in the adrenal medulla of an *Rb*-DKO chimera; normal medulla (M) and cortex (C) are indicated. (**B**) High magnification of a section through the retina of an *Rb*-DKO chimera with normal outer nuclear (O), bipolar cell (B), and ganglionic cell (G) layers. (**C**) Section of whole eye of an *Rb*-DKO chimera showing a cataract (C) in the lens (L) and normal retina (R). (**D**) Section of normal eye of a newborn control chimera with lens (L) and retina (R) indicated. (**E**) Section of eye of a newborn *Rb*-DKO chimera showing abnormal lens (L) architecture and an early cataract (C); retina (R) is also noted. (**F**) Higher magnification view of area boxed in panel E. Disorganized lens fiber cells are shown at the bottom, dying cells with pyknotic nuclei in the middle and cellular debris above. (**G**) Section of an *Rb*-DKO chimera liver showing cells with abnormally enlarged nuclei (arrows). (**H**) Normal cerebellum from a control chimera. The granular cell (G), molecular (M) and Purkinje cell layers (P) and normal Purkinje cells (arrow) are indicated. (**I**) Abnormal cerebellum in an *Rb*-DKO chimera. Ectopic and enlarged Purkinje cells are shown (arrows) along with reduced granular cell layer (G). Magnification: panels A, B and F–I, 200×; panels C–E, ~50×.

smears from the *Rb*-DKO chimeras further confirmed that the cells producing the 129/sv-specific β-globin were indeed mature erythrocytes (Figure 3B). Notably, there was no increase in the percentage of nucleated RBC in these animals compared with controls. Thus, it is clear that *Rb*-deficient erythroid cells, whose development is inhibited in an *Rb* homozygous mutant embryo, can develop normally when in the environment of wild-type cells.

Histological and pathological consequences of Rb deficiency

The analysis described above indicates a surprisingly large percentage of *Rb*-deficient cells in many tissues of the adult chimeras. However, these data do not address whether the development of these mutant cells occurred normally. In particular, it was possible that the *Rb*-deficient cells might be defective in reaching or maintaining endstage differentiation which could produce pathological and tumorigenic consequences in some tissues. Therefore, we undertook a detailed histopathological characterization of a number of tissues in the chimeric animals to determine whether their development was, in fact, fully normal. For all of the tissues examined by GPI analysis, a sample was fixed, processed and examined by standard histological methods.

For the majority of tissues analyzed, the *Rb*-DKO chimeras were indistinguishable from controls. In four tissue types, however, the presence of *Rb*-deficient cells produced obvious abnormalities. When the adrenal medullae of four *Rb*-DKO chimeras were examined histologically, all four were found to contain multiple small nodules of hyperchromatic cells, indicative of preneoplastic lesions (Figure 4A). In addition, in six of eight animals examined, areas of the cerebellar cortex had displaced Purkinje cells, most of which had enlarged nuclei (Figure 4H and I). In the affected cortical areas, the granular cell layer was also abnormally thin (Figure 4H and I). This defect in cerebellar differentiation is interesting considering the widespread neuronal cell death seen in *Rb* homozygous mutant embryos. The livers of several *Rb*-DKO chimeras also contained hepatocytes with abnormally enlarged nuclei (Figure 4G).

Finally, the most consistent abnormality observed in the *Rb*-DKO chimeras involved the lens of the eye: each of these animals developed bilateral cataracts (Figure 4C). These cataracts were characterized by posterior migration of the lens epithelium associated with bladder cell formation and, in some cases, calcification. Most animals showed evidence of cataract formation at the time the eyes opened (~1 week of age), and several newborns examined histologically already had obvious lesions in the lens. The lenses of chimeric newborns were highly disorganized and contained large numbers of pyknotic cells, indicative of apoptosis (Figure 4E and F). None of these defects were observed in control chimeras made from heterozygous ES cells. Despite a measurable contribution of *Rb*-deficient cells to the eyes, including crude retinal preparations (Figure 2A and B), we have not observed any evidence of retino-blastoma in the *Rb*-DKO chimeras. Moreover, the retinal architecture appeared normal (Figure 4B). Thus, the absence of *Rb* function alone does not lead to the outgrowth of clones of transformed cells in the mouse retina.

Pituitary tumorigenesis

At 3–4 months of age the *Rb*-DKO chimeras exhibited severe wasting and became moribund. Autopsy revealed severe adenocarcinoma of the pituitary, the same tumor observed in animals heterozygous for the Rb^{x3t} mutation (Jacks *et al.*, 1992). Immunohistochemical analysis performed on three of these tumors indicated that they arose from the intermediate lobe of the pituitary. The tumors stained positively with antibodies directed against the hormones α-melanocyte stimulating hormone (MSH), β-endorphin and adrenocorticotropic hormone (ACTH) (all produced in the intermediate lobe) and not for markers specific for other lobes of the pituitary including thyroid stimulating hormone (TSH) and prolactin (Figure 5, data not shown). This pattern of staining was also observed with pituitary tumors from Rb^{x3t} heterozygotes (data not shown).

The average age at which *Rb*-DKO chimeras manifested the effects of pituitary tumorigenesis was ~4 months (Figure 6). All 10 chimeras that were not sacrificed at earlier ages for other purposes eventually developed this tumor. Thus, the latency of tumorigenesis observed in the *Rb*-DKO chimeras is greatly reduced relative to the Rb^{x3t} heterozygotes, most of which succumb to pituitary tumors at ~9–10 months of age (Figure 6). Interestingly, in both *Rb* heterozygotes and the *Rb*-DKO chimeras, the time window during which most animals are affected is remarkably narrow and well-defined (Figure 6). Thus, it appears that the absence of *Rb* accelerates the death of the *Rb*-DKO chimeras (relative to *Rb* +/− mice) caused by pituitary tumorigenesis. Although we have also observed preneoplastic lesions in the adrenal glands of some *Rb*-DKO chimeras, it is noteworthy that there are relatively few tissues in which homozygous inactivation of *Rb* causes oncogenic transformation.

Discussion

Mutant alleles of the *RB* gene are found in a variety of human tumors, including the retinal tumors in which this gene was first characterized, osteosarcomas, carcinomas of the lung, bladder and breast (Friend *et al.*, 1986, 1987; Fung *et al.*, 1987; W.H.Lee *et al.*, 1987; Harbour *et al.*, 1988; E.Y.-H.P.Lee *et al.*, 1988; T'Ang *et al.*, 1988; Yokota *et al.*, 1988; Varley *et al.*, 1989; Bookstein *et al.*, 1990b; Hensel *et al.*, 1990). For this reason, the *RB* gene has been thought to function as an important negative regulator of cell proliferation in these various tissues. An even more widespread involvement in growth regulation has been suggested by the almost ubiquitous expression of this gene in the tissues of the mouse (Bernards *et al.*, 1989; Szekely *et al.*, 1992) and the growth inhibitory effects of exogenous *RB* expression (Huang *et al.*, 1988; Bookstein *et al.*, 1990a; Qin *et al.*, 1992). The RB protein is found to undergo phosphorylation in mid/late G_1 (Buchkovich *et al.*, 1989; Chen *et al.*, 1989; DeCaprio *et al.*, 1989) and this phosphorylation has been connected with the functioning of cyclins and associated kinases (Lees *et al.*, 1991; Lin *et al.*, 1991; Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993) Moreover, pRb has been shown to associate with a number of DNA tumor virus oncoproteins (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989) and cellular transcription factors, most notably E2F (Chellappan *et al.*, 1991; Rustgi *et al.*,

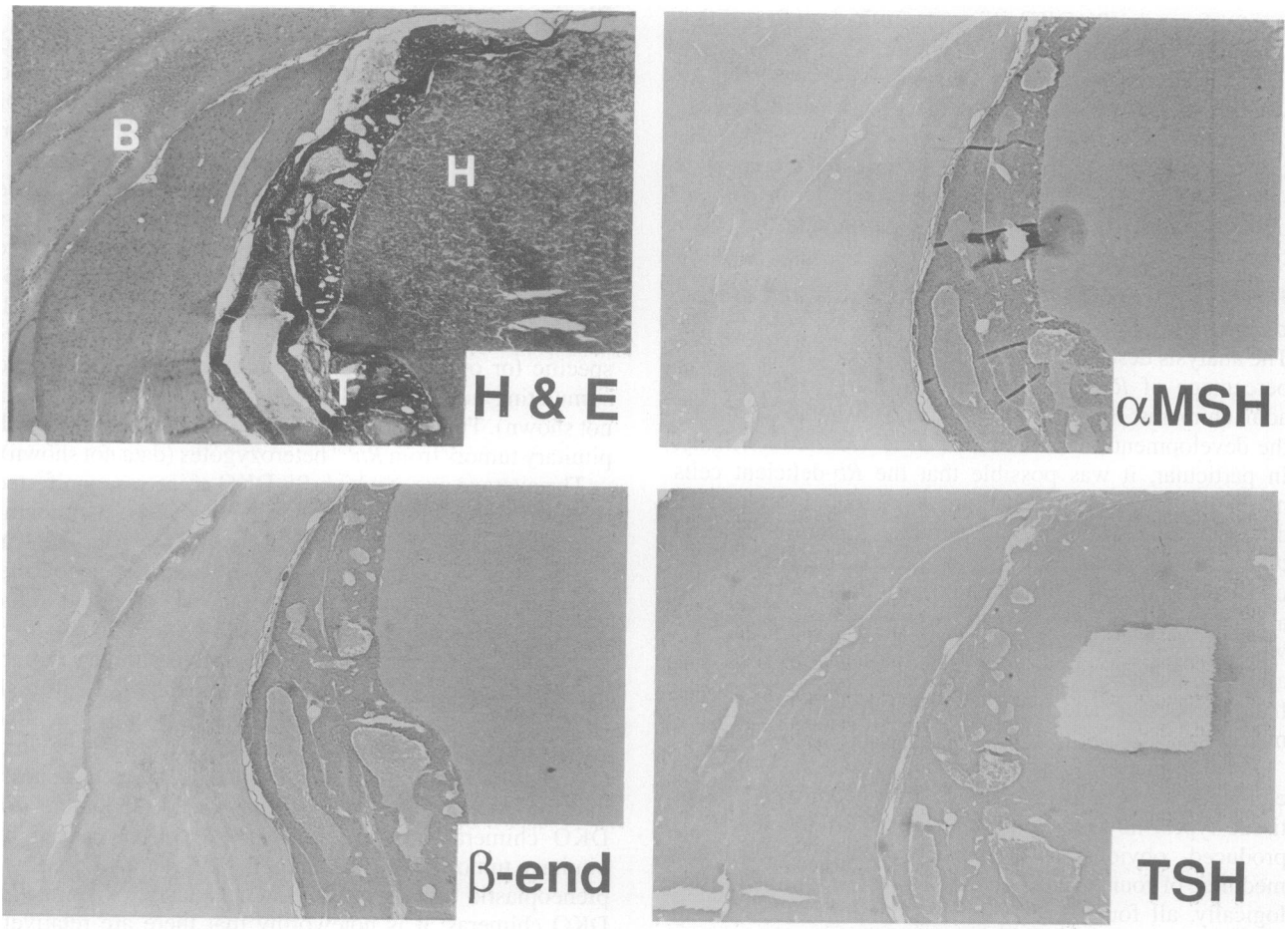

Fig. 5. Immunohistochemistry of pituitary tumors. Section of pituitary from an *Rb*-DKO chimera stained with hematoxylin and eosin (H & E) showing tumor (T), brain (B) and a large hematoma (H). Tumor tissues stain positively with antibodies against the hormones α-MSH, β-endorphin (β-end) and ACTH (not shown) produced in the intermediate lobe of the pituitary and not with antibodies for thyroid stimulating hormone (TSH) or other markers of the anterior pituitary, including prolactin (not shown).

1991; Gu *et al.*, 1993; Hagemeier *et al.*, 1993). Taken together, these various lines of evidence imply that pRb regulates progression through a critical point in the cell growth cycle.

The resulting model of pRb as an important controller of proliferation and transformation in a wide variety of cell types is difficult to reconcile with the observations presented here. The present work describes the creation of chimeric mice, many of whose tissues are morphologically normal yet contain large proportions of *Rb*-deficient cells. These mice are largely developmentally normal and survive until 3–4 months *post partum*, at which time they die from the effects of adenocarcinomas of the pituitary. In a separate series of experiments, we have also been able to produce apparently normal *Rb*-deficient B and T lymphocytes using the RAG-2 blastocyst complementation assay (Chen *et al.*, 1993). Thus, the accumulated data suggest that *Rb* is dispensable for the development and maintenance of the differentiated state of most cell types in the mouse. This extends the earlier observations which showed that mouse embryos homozygous for an *Rb* mutation develop normally until day 13 of gestation, at which point they die, apparently because of a defect in erythropoiesis (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). It is important to note that while the data

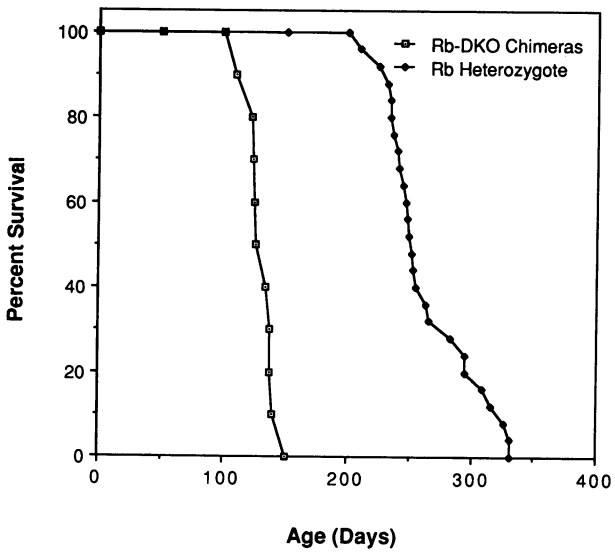

Fig. 6. Viability of *Rb*-DKO chimeras. Graph summarizes the viability of 10 *Rb*-DKO chimeras and 25 animals heterozygous for a germline mutation in *Rb*. Heterozygotes are inbred on the 129/Sv genetic background. Data points reflect the time at which animals became moribund and were sacrificed due to the effects of intermediate lobe pituitary tumors.

presented here suggest that *Rb*-deficient cells are capable of contributing to all tissues of the adult mouse, the ability of these cells to differentiate into every cell type within a given tissue cannot be determined at this time. Therefore, it remains possible that there are certain cells in the animal that cannot be formed in the absence of *Rb* function. Moreover, the survival of embryos that are composed largely of *Rb*-deficient cells may still require the contribution of wild-type cells to one or more critical cell lineages during development.

One of the more unexpected results of this analysis was the high percentage of *Rb*-deficient erythrocytes in the peripheral blood of chimeric animals. Due to incomplete differentiation *in vitro* of erythroid precursors isolated from the livers of *Rb* homozygous mutant embryos, we had previously suggested that the requirement for *Rb* in erythropoiesis was cell autonomous; that is, the function of the *Rb* gene was required within erythroid precursor cells in order for them to differentiate normally (Jacks *et al.*, 1992). However, we now consider it more likely that the absence of *Rb* function does not affect erythropoiesis by directly inhibiting differentiation of erythroid cells *per se* but, rather, by affecting the ability of other cell types to create an environment allowing survival and differentiation. For example, the absence of *Rb* function might prevent stromal cells from producing one or more critical growth, differentiation or survival factors essential for normal erythroid differentiation. In the chimeras, the wild-type cells derived from the host embryo could 'rescue' the differentiation of the mutant cells by supplying the necessary extracellular factor(s) to the microenvironment. The previously described inefficient *in vitro* differentiation of *Rb*-deficient precursors might be explained by a pre-existing defect due to their failure to receive appropriate environmental signals prior to their removal from the embryo. Furthermore, the apparently normal development of other *Rb*-deficient cell types could also be affected by the presence of wild-type cells in the chimeras.

According to Knudson's two-hit model of retinal tumorigenesis, the development of human retinoblastoma is dependent on two rate-limiting steps (Knudson, 1971), which are now understood to be the successive inactivation of two copies of the *Rb* gene (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). Since mice heterozygous for an *Rb* mutation do not develop retinoblastoma, we and others have proposed that the loss of function of *Rb* is not sufficient to transform the relevant cell type in the mouse retina (Clarke *et al.*, 1992; Jacks *et al.*, 1992; Lee *et al.*, 1992). Alternatively, the absence of retinoblastoma in these animals could reflect the relatively small number of susceptible cells in the retina of the mouse, which would be expected to decrease the likelihood of the second hit in Knudson's model. Having found that the *Rb*-DKO chimeras (in which a measurable fraction of retinal cells are already homozygous for an *Rb* mutation) also do not develop retinoblastoma, we now strongly favor the notion that the relevant cell type in the mouse is refractory to the loss of *Rb* function. This implies that the transformation of this cell type in the mouse requires additional genetic events and may explain why sporadic retinoblastoma has never been reported in mice (or indeed in any other species besides our own). Mice expressing SV40 T antigen do develop retinoblastoma at high frequency (Windle *et al.*, 1990; al-Ubaidi *et al.*, 1992), suggesting that some of the multiple activities of this viral oncoprotein beyond its ability to bind and sequester pRb (DeCaprio *et al.*, 1988) contribute to retinal cell transformation.

Mice heterozygous for the Rb^{x3t} mutation are highly predisposed to intermediate lobe pituitary tumors (Jacks *et al.*, 1992). Tumorigenesis appears to require the inactivation of the wild-type *Rb* allele, and the average age of sacrifice (from the effects of the tumor) is ~10 months. In the *Rb*-DKO chimeras, the rate of tumorigenesis is increased; these animals become moribund from the effects of the pituitary tumors by 4 months of age. Thus, as predicted from Knudson's model (Knudson, 1971), the removal of a rate-limiting step (the inactivation of the wild-type allele of *Rb*) accelerates the rate of tumor development. This increased rate could result from the more rapid formation of a single tumor focus or the combined effects of several distinct foci. By sacrificing animals at progressively earlier ages, it should be possible to determine the number of foci that contribute to tumor growth in both the heterozygotes and the *Rb*-DKO chimeras. The strikingly precipitous decline in the survival of the *Rb*-DKO chimeras during a narrow window of time indicates that few, if any, additional stochastic events are required for the transformation of the cells of the intermediate lobe of the pituitary.

Finally, given the frequent mutation of the *Rb* gene in human cancer and the proposed importance of this gene in cell cycle regulation (Weinberg, 1992), we had anticipated that seeding an animal with large numbers of *Rb*-deficient cells would result in widespread tumorigenesis but found that tumorigenesis is limited to the pituitary gland and the adrenal medulla. In order to produce more profound effects on tumorigenesis (and development) it may be necessary to inactivate simultaneously two or more members of the *Rb* family of genes which now includes the genes encoding p107 (Ewen *et al.*, 1991) and p130 (Cobrinik *et al.*, 1993; Hannon *et al.*, 1993; Li *et al.*, 1993).

Materials and methods

Creation of Rb-DKO ES cells

Heterozygous ES cells (Jacks *et al.*, 1992) were electroporated with an *Rb* targeting vector containing a hygromycin resistance gene in place of the neomycin resistance gene of the original targeting vector, described in Jacks *et al.* (1992). The hygromycin gene is under the control of the *PGK* promoter and polyadenylation sequences (te Riele *et al.*, 1990). The conditions for electroporation in the second round of gene targeting were identical to the first (Jacks *et al.*, 1992). Following electroporation, cells were plated in the absence of a feeder layer, and selected with 300 μg/ml G418, 125 μg/ml hygromycin and 2 μM gancyclovir. Colonies were selected and expanded for DNA analysis and subsequent blastocyst injection.

Southern blot analysis

Preparation of genomic DNA and Southern blotting were performed exactly as described (Jacks *et al.*, 1992) using ^{32}P-labeled probes indicated in Figure 1A.

Generation of chimeric mice

Blastocyst injection and creation of chimeric mice were performed as described (Bradley, 1987) except that eight to 10 ES cells were injected per blastocyst.

Histological analysis of tissues

Tissues were surgically removed, fixed in Bouin's solution, dehydrated in graded alcohol solutions, embedded in paraffin, sectioned at 6 μm and stained with hematoxylin and eosin.

Immunohistochemistry

Tissues fixed in Bouin's solution were stained with polyclonal antibodies to prolactin and TSH (generously provided by Salvatore Raiti of the National Hormone and Pituitary Program at the University of Maryland School of Medicine with the National Institute of Diabetes and Digestive and Kidney Diseases), β-endorphin, α-MSH and ACTH (BioGenex Laboratories). Immunohistochemistry was performed using appropriate secondary antibodies (BioGenex Laboratories) and a biotin-streptavidin-peroxidase-based staining kit with a diaminobenzidine substrate (BioGenex Laboratories)

Retinal isolation

Crude retinal isolates were obtained by removing the cornea and lens with a circumferential incision behind the limbus. Retinal tissue was first excised along with the attached optic nerve and further dissected away from it.

Preparation of red blood cell fractions

Whole blood in anticoagulant was diluted 2-fold in phosphate buffered saline, layered onto two volumes of Ficoll–Hypaque (Histopaque-1077, Sigma) and centrifuged at 3500 *g* for 20 min at room temperature. The pellet, enriched for RBC, was collected; platelets remain at the top of the gradient while lymphocytes and monocytes are enriched at the interphase.

GPI assay

Separation and detection of GPI isoenzymes was performed essentially as described (Bradley, 1987). Titan III Zip Zone cellulose acetate plates (Helena Laboratories) were soaked in Tris–glycine buffer (25 mM Tris, 200 mM glycine, pH 8.5) for 20 min prior to application of samples. Samples were prepared by diluting tissues with ~10 vol of water, and were then crudely homogenized; cells were then lysed by three rounds of freezing and thawing. Most samples were then further diluted 10-fold with distilled water. Purified RBC were diluted a total of 1:5. Samples were electrophoresed in triplicate in a Zip Zone chamber (Helena Laboratories) for 1.5 h at 150 V at 4°C, and stained as described (Bradley, 1987). Relative levels of the two GPI isoforms were quantified by densitometry with an LKB 2222-010 UltroScan XL linear densitometer. Tissues analyzed included cerebellum, cerebrum, medulla oblongata, crude retinal isolate, epididymis, RBC, lung, tongue, bone marrow, testis, liver, uterus, optic nerve, thymus, diaphragm, colon, eye, lymphocytes and monocytes, spleen, tail, cecum, stomach, bladder, atrium, ventricle, salivary gland, small intestine, ovary, kidney, leg muscle, seminal vesicle, trachea/thyroid, prostate, esophagus, pancreas, fifth cranial nerve and adrenal gland.

Hemoglobin assay

Cellulose acetate electrophoresis of cystamine-modified hemoglobins was performed essentially as described (Whitney, 1977; Pevny *et al.*, 1991). Purified RBC were added to 75 ml of cystamine lysis buffer (12.5 mg/ml cystamine dihydrochloride, 1 mM dithiothreitol, 0.55% ammonium hydroxide) and agitated to lyse the RBC. Samples were applied to Titan III cellulose acetate plates (Helena Laboratories) (previously soaked for 10 min in an electrophoresis buffer of 0.18 M Tris, 0.10 M boric acid, 0.002 M EDTA) and run for 40 min at 300 V. The plates were placed in staining solution (1% Ponceau S, 5% trichloroacetic acid) for 10 min and rinsed in three changes of 5% acetic acid for 10 min each. Plates were then immersed in 100% ethanol for 10 min, then in clearing solution (30% acetic acid, 70% methanol) for 5 min, and finally baked at 55°C for 5 min.

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References

al-Ubaidi,M.R., Font,R.L., Quiambao,A.O., Keener,M.J., Liou,G.I., Overbeek,P.A. and Baehr,W. (1992) *J. Cell Biol.*, **119**, 1681–1687.

Bernards,R. *et al.* (1989) *Proc. Natl Acad. Sci. USA*, **86**, 6474–6478.

Bookstein,R., Rio,P., Madreperla,S.A., Hong,F., Allred,C., Grizzle,W.E. and Lee,W.-H. (1990a) *Proc. Natl Acad. Sci. USA*, **87**, 7762–7766.

Bookstein,R., Shew,J.Y., Chen,P.L., Scully,P. and Lee,W.H. (1990b) *Science*, **247**, 712–715.

Bradley,A. (1987) In Roberton,E.J. (ed), *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. IRL Press, Oxford, pp. 113–152.

Buchkovich,K., Duffy,L.A. and Harlow,E. (1989) *Cell*, **46**, 447–456.

Chellappan,S.P., Hiebert,S., Mudryj,M., Horowitz,J.M. and Nevins,J.R. (1991) *Cell*, **65**, 1053–1061.

Chen,J., Gorman,J., Stewart,V., Williams,B., Jacks,T. and Alt,F.W. (1993) *Curr. Biol.*, **3**, 405–413.

Chen,P.-L., Scully,P., Shew,J.-Y., Wang,J.Y.J. and Lee,W.-H. (1989) *Cell*, **58**, 1193–1198.

Clarke,A.R., Maandag,E.R., van Roon,M., van der Lugt,N.M.T., van der Valk,M., Hooper,M.L., Berns,A. and te Riele,H. (1992) *Nature*, **359**, 328–330.

Cobrinik,D., Whyte,P., Peeper,D.S., Jacks,T. and Weinberg,R.A. (1993) *Genes Dev.*, **7**, 2392–2404.

DeCaprio,J.A., Ludlow,J.W., Figge,J., Shew,J.-Y., Huang,C.-M., Lee,W.H., Marsilio,E., Paucha,E. and Livingston,D.M. (1988) *Cell*, **54**, 275–283.

DeCaprio,J.A., Ludlow,J.W., Lynch,Y., Furukawa,J., Griffin,H., Piwnica-Worms,C.-M., Huang,C.-M. and Livingston,D.M. (1989) *Cell*, **58**, 1085–1095.

Dowdy,S.F., Hinds,P.W., Louie,K., Reed,S.I., Arnold,A. and Weinberg,R.A. (1993) *Cell*, **73**, 499–511.

Dyson,N., Howley,P.M., Munger,K. and Harlow,E. (1989) *Science*, **243**, 934–937.

Ewen,M.E., Xing,Y., Lawrence,J.B. and Livingston,D.M. (1991) *Cell*, **66**, 1155–1164.

Ewen,M.E., Sluss,H.K., Sherr,C.J., Matsushime,H., Kato,J.-y. and Livingston,D.M. (1993) *Cell*, **73**, 487–497.

Friend,S.H., Bernards,R., Rogelj,S., Weinberg,R.A., Rapaport,J.M., Albert,D.M. and Dryja,T.P. (1986) *Nature*, **323**, 643–646.

Friend,S.H., Horowitz,J.M., Gerber,M.R., Wang,X.F., Bogenmann,E., Li,F.P. and Weinberg,R.A. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 9059–9063.

Fung,Y.K., Muphree,A.L., T'Ang,A., Qian,J., Hinrichs,S.H. and Benedict,W.F. (1987) *Science*, **236**, 1657–1661.

Gossler,A., Doetschman,T., Korn,R., Serfling,E. and Kemier,R. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 9065–9069.

Gu,W., Schneider,J.W., Condorelli,G., Kaushal,S., Mahdavl,V. and Nadal-Ginard,B. (1993) *Cell*, **72**, 309–324.

Hagemeier,C., Bannister,A.J., Cook,A. and Kouzarides,T. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 1580–1584.

Hannon,G.J., Demetrick,D. and Beach,D. (1993) *Genes Dev.*, **7**, 2378–2391.

Harbour,J.W., Lai,S.-L., Whang-Pend,J., Gazdar,A.F., Minna,J.D. and Kaye,F.J. (1988) *Science*, **241**, 353–356.

Hensel,C.H., Hsieh,C.L., Gazdar,A.F., Johnson,B.E., Sakaguchi,A.Y., Naylor,S.L., Lee,W.-H. and Lee,E.Y.-H.P. (1990) *Cancer Res.*, **50**, 3067–3072.

Hinds,P.W., Mittnacht,S., Dulic,V., Arnold,A., Reed,S.I. and Weinberg,R.A. (1992) *Cell*, **70**, 993–1006.

Huang,H.J., Yee,J.-K., Shew,J., Chen,P.-L., Bookstein,R., Friedmann,T., Lee,E.Y.-H.P. and Lee,W.-H. (1988) *Science*, **242**, 1563–1566.

Jacks,T., Fazeli,A., Schmidt,E., Bronson,R., Goodell,M. and Weinberg,R. (1992) *Nature*, **359**, 295–300.

Knudson,A.G. (1971) *Proc. Natl Acad. Sci. USA*, **68**, 820–823.

Knudson,A.G. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 10914–10921.

Lee,E.Y.-H.P., To,J., Shew,J.Y., Bookstein,R., Scully,P. and Lee,W.-H. (1988) *Science*, **241**, 218–221.

Lee,E.Y.-H.P., Chang,C.-Y., Hu,N., Wang,Y.-C.J., Lai,C.-C., Herrup,K. and Lee,W.-H. (1992) *Nature*, **359**, 288–295.

Lee,W.H., Bookstein,R., Hong,F., Yong,L.J., Shew,J.Y. and

Lee,E.Y.-H.P. (1987) *Science*, **235**, 1394–1399.
Lees,J.A., Buchkovich,K.J., Marshak,D.R., Anderson,C.W. and Harlow,E. (1991) *EMBO J.*, **10**, 4279–4289.
Li,Y., Graham,C., Lacy,S., Duncan,A.M.V. and Whyte,P. (1993) *Genes Dev.*, **7**, 2366–2377.
Lin,B.T.-Y., Gruenwald,S., Morla,A.O., Lee,W.-H. and Wang,J.Y.J. (1991) *EMBO J.*, **10**, 857–864.
Lyon,M.F. and Searle,A.G. (1990) *Genetic Variants and Strains of the Laboratory Mouse*. Oxford University Press, Oxford.
Mansour,S.L., Thomas,K.R. and Capecchi,M.R. (1988) *Nature*, **336**, 348.
Pevny,L., Simon,M.C., Robertson,E., Klein,W.H., Tsai,S.-F., D'Agati,V., Orkin,S.H. and Costantini,F. (1991) *Nature*, **349**, 257–260.
Qin,X., Chittenden,T., Livingston,D.M. and Kaelin,J.W.G. (1992) *Genes Dev.*, **6**, 953–964.
Rustgi,A.K., Dyson,N. and Bernards,R. (1991) *Nature*, **352**, 541–544.
Szekely,L., Jiang,W.-Q., Bulic-Jakus,F., Rosen,A., Ringertz,N., Klein,G. and Wiman,K.G. (1992) *Cell Growth Diff.*, **3**, 149–156.
T'Ang,A., Varley,J.M., Chakraborty,S., Murphree,A.L. and Fung,Y.-K.T. (1988) *Science*, **241**, 1797–1800.
te Riele,J., Robanus Maandag,E., Clarke,A., Hooper,M. and Berns,A. (1990) *Nature*, **348**, 649–651.
Varley,J.M., Armour,J., Swallow,J.E., Jeffreys,A.J., Ponder,B.A., T'Ang,A., Fung,Y.K., Brammar,W.J. and Walker,R.A. (1989) *Oncogene*, **4**, 725–729.
Weinberg,R.A. (1992) *Cancer Surv.*, **12**, 43–57.
Whitney,J.B.,III (1977) *Biochem. Genet.*, **16**, 667–672.
Whyte,P., Buchkovich,K.J., Horowitz,J.M., Friend,S.J., Raybuck,M., Weinberg,R.A. and Harlow,E. (1988) *Nature*, **334**, 124–129.
Windle,J.J., Albert,D.A., O'Brien,J.M., Marcus,D.M., Disteche,C.M., Bernards,R. and Mellon,P.L. (1990) *Nature*, **343**, 665–668.
Yokota,J. *et al.* (1988) *Oncogene*, **3**, 471–475.

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